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THE BIOCHEMISTRY OF SEMEN

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The Biochemistry of Semen

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WITH 7 PLATES AND 16 TEXT FIGURES

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PREFACE

When I took up my studies on semen in 1944, on behalf of the Agricultural Research Council, I became painfully aware of the fact that information on the physiology of semen, its chemical aspects in particular, is rather difficult to come by; the older observations and records being hidden away in books and journals not readily accessible in any but the best equipped libraries, and moreover, scattered throughout an exceptionally wide range of publications, which embrace disciplines as far apart as say, agriculture, urology and cytology. Judging from numerous requests for information, received from fellow workers in the field, biochemists, clinicians, zoologists and veterinary officers alike, the absence of a fairly comprehensive and up-to-date treatise on the chemical physiology of semen must have proved a serious handicap to many in their scientific and practical pursuits. Therefore, I accepted gladly the invitation to write this book; having agreed to produce but a 'little book', I have often found it rather irksome to condense the vast mass of data into the allotted space; had it not been for the encouragement and ready help of colleagues—my wife not least among them, the task would have been even more burdensome.

Biochemistry of semen is a relatively modern, but rapidly expanding, field of physiology; consequently, many of our present views, particularly as regards the biological significance of various chemical constituents of semen, may have to be revised or modified in the near future. That being so, I like to look upon this book, or at any rate, those parts of it which deal with the newer, still fluid concepts, as something in the nature of an Interim Report, designed to furnish information and to convey ideas emerging from the state of knowledge as available at the time of writing, however imperfect that may be. In presenting the recently acquired evidence, I have tried to render justice to developments in the sphere of mammalian as

well as non-mammalian physiology, selecting examples from species as far apart as man and the sea-urchins, and occasionally, introducing plants as well. I have done my best to distinguish between established fact and tentative hypothesis, and, as far as possible, have refrained from the tendency, currently prevalent among workers in this field, to assign to every newly discovered chemical constituent of semen a major role in the process of fertilization.

I wish to acknowledge gratefully the help of those who gave me permission to reproduce plates and figures. In particular I wish to extend my thanks to Dr. C. R. Austin (Sydney), Dr. J. L. Hancock (Cambridge) and the Cambridge University Press for Plate I, to Prof. L. H. Bretschneider and Dr. Woutera van Iterson (Utrecht) and the Nederland Academy of Science for Plate II, to the Royal Society for Plate III, to Lord Rothschild (Cambridge) for Plate IV and for reading the manuscript, to the Royal Society of Edinburgh for Fig. 2, to Dr. E. Blom (Copenhagen) and the *Skandinavisk Veterinärtidskrift* for Fig. 3, to Dr. C. Huggins (Chicago) and the Harvey Society of New York for Fig. 5, to Dr. L. Jacobsson (Göteborg) and the *Acta Physiologica Scandinavica* for Fig. 11, and to the Cambridge University Press, Messrs. Churchill and Messrs. Macmillan for permission to reproduce Figs. 6–10, 12–14 and 16, from the *Biochemical Journal*, the *Journal of Agricultural Science*, and *Nature*, and Plate IV, from the Ciba Foundation Symposium on *Mammalian Germ Cells*. I should also like to thank Miss P. A. Northrop for helping me in the preparation of the typescript.

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INTRODUCTION

Before I decided to embark upon the business of studying the metabolism of semen, my interest used to centre on very different biochemical problems; earlier on, in the laboratory of J. K. Parnas, I was youthfully grappling with the intricacies of intermediary carbohydrate metabolism in muscle, blood and yeast; later on, at the Molteno Institute, in happy association with D. Keilin, we were investigating the nature and function of metalloprotein enzymes in plant and animal tissues. When confronted with the opportunity of an extensive study of spermatozoa, I did not hesitate to give up my former pursuits in order to devote myself to experiments involving biological material which offers the investigator a chance, almost unique so far as mammalian tissues are concerned, of correlating chemical and metabolic findings with clearly defined and highly specific criteria of physiological activity, such as the motility and fertilizing capacity of the spermatozoa. Among other peculiarities which make semen such a fascinating and attractive object of study is that it represents an animal tissue with but a single type of cells, the spermatozoa, freely suspended in a fluid medium of some complexity, the seminal plasma, and not subject to cellular growth, division or multiplication; thus, making it feasible to express all one's metabolic measurements directly in terms of cell numbers, without recourse to cumbersome and often unreliable standards such as dry weight of tissue, nitrogen content, or indeed, any other of the commonly used metabolic indices. From the purely practical point of view, which matters greatly, the ability of spermatozoa to 'survive', i.e. retain their remarkable properties under conditions of long-term storage *in vitro*, is of great importance. This in turn, gives one a chance of exploring at will and under well-defined conditions *in vitro*, the intricate chemical mechanism underlying the viability, and ultimately, the senescence, of living animal cells.

So far as the nutrition of spermatozoa is concerned, semen resembles more a suspension of microorganisms in a nutrient medium, than other animal tissues which rely for their nutrients

on the blood supply. Nature has endowed the spermatozoa with the means of very efficient utilization of extraneous sources of energy, such as are accessible to the sperm cells either in their natural environment, the seminal plasma, or in the artificial storage media.

As will be evident from what follows later, the present century has witnessed much that is new in the field of semen biochemistry. By and large, however, the situation is not very different from what it was two centuries ago, when Charles Bonnet addressed the following remarks about spermatozoa to Spallanzani:

'They are, of all animalculi of liquids, those which have most excited my curiosity: the element in which they live, the place of their abode, their figure, motion, their secret properties; all, in a word, should interest us in so singular a kind of minute animated beings. How are they found there, how are they propagated, how are they developed, how are they fed, and what is their motion? What becomes of them when the liquid they inhabit is reabsorbed by the vessels and returned to the blood? Why do they appear only at the age of puberty; where did they exist before this period? Do they serve no purpose but to people the fluid where they are so largely scattered? How far are we from being able to answer any of these questions! And how probable it is, that future age will be as ignorant of the whole, as our own!'

CHAPTER I

The Two Components of Semen: Spermatozoa and Seminal Plasma

Spermatozoa. Spermatogenesis and sperm 'ripening'. Sperm transport in the female reproductive tract and 'capacitation'. Structural and chemical characteristics of the sperm-head, middle-piece and tail.

Seminal plasma. Secretory function of male accessory glands. Prostatic secretion. Seminal vesicle secretion. Physiological significance of seminal plasma. Prostaglandin, vesiglandin, and certain other pharmacodynamically active substances. Coagulation and liquefaction.

'WHOLE semen' as ejaculated, generally appears as a viscous, creamy, slightly yellowish or greyish fluid, and consists of spermatozoa or 'sperm', suspended in the fluid medium, called seminal plasma; its composition depends in the first place, on the proportion of sperm and plasma, and is further determined by the size, storage capacity, and secretory output of several different organs which comprise the male reproductive tract. The volume of the ejaculate and the concentration of spermatozoa or the 'sperm density' in ejaculated semen, vary widely from one species to another, as seen from Table 1. A single ram ejaculate for instance, amounts to 0·7–2 ml. only, but is distinguished by a very high sperm density, 2–5 million per μl. semen; when subjected to high-speed centrifugation, ram semen separates, on the average, into about two-thirds of seminal plasma and one-third of firmly packed sperm. Boar semen ejaculates on the other hand, may reach a volume of as much as 500 ml.; this is not due to spermatozoa, but to the seminal plasma generated in very capacious accessory organs (Plate III); a sperm density not exceeding 100,000 cells/μl. is quite usual for boars, and even lower sperm densities would still be regarded as normal. In man, the average volume of a single ejaculate is about 3 ml., but the sperm density is frequently less than 100,000 cells/μl., so that only a small portion of the ejaculate, much less than 10%, is represented by the sperm and the rest is seminal plasma.

TABLE 1. *Species differences in volume and sperm density of ejaculated semen*

Species	*Volume of single ejaculate*		*Sperm density in semen*	
	Normal variations (ml.)	Most common value (ml.)	Normal variations (sperm/μl.)	Average value (sperm/μl.)
Ass	10–80	50	200 000– 600 000	400 000
Bat		0·05	5 000 000–8 000 000	6 000 000
Boar	150–500	250	25 000– 300 000	100 000
Bull	2–10	4	300 000–2 000 000	1 000 000
Cock	0·2–1·5	0·8	50 000–6 000 000	3 500 000
Dog	2–15	6	1 000 000–9 000 000	3 000 000
Fox	0·2–4	1·5	30 000– 250 000	70 000
Man	2–6	3·5	50 000– 150 000	100 000
Rabbit	0·4–6	1	100 000–2 000 000	700 000
Ram	0·7–2	1	2 000 000–5 000 000	3 000 000
Stallion	30–300	70	30 000– 800 000	120 000
Turkey	0·2–0·8	0·3		7 000 000

The two components of semen, sperm and seminal plasma, differ in their origin, composition and function, and must be considered separately, in much the same sense as for instance, blood corpuscles and blood plasma.

Early investigators of semen were, not unnaturally, fascinated by the spermatozoa, and, with the aid of such optical instruments as were available to them, concentrated their efforts upon the elucidation of the structural details of spermatozoa. But it is very much to their credit that they have not entirely neglected the seminal plasma. Thus, the letter in which Antoni van Leeuwenhoek reported in 1677 to the Royal Society on sperm motion, also contains the earliest description of spermin crystals in the seminal plasma. Louis Nicolas Vauquelin, the author of the first treatise on the chemical composition of semen (*Expériences sur le sperme humain*, 1791), fully appreciated the separate existence of sperm and seminal plasma; the same was true of his followers, among them Friedrich Miescher, whose collected writings, published in 1897, contained much new information concerning not only spermatozoa but the seminal plasma as well.

The work of Miescher and his contemporaries, however, dealt largely with fish spermatozoa, and even during the early decades of

the present century, research on semen was, on the whole, confined to fish and generally to animals in which fertilization takes place externally, and which provide the experimental material in conveniently large quantities. The tardy progress of research on the spermatozoa and seminal plasma of birds and mammals was due in the main to the difficulty of securing enough material for experimental purposes; however, more rapid advances were made soon after Elie Ivanov (1907) and several other pioneers in the field of artificial insemination, perfected the technique of semen collection from domestic animals. The widening practice of artificial insemination for breeding purposes on a large scale, early revealed the need for improved standards of sperm evaluation and in this way provided a powerful stimulus for morphological as well as chemical investigations on semen. At the same time, clinical enquiries into the causative and diagnostic aspects of human infertility also pointed to serious gaps and deficiencies in the knowledge of the physiology of human semen.

The last two decades have witnessed rapid advances in the application of laboratory methods of semen analysis to the study of the manifold causes underlying male sterility and subfertility, and there is a steadily increasing number of publications on this subject, which has been comprehensively reviewed on several occasions. Some of these articles and monographs refer specifically to man (Joël, 1942; Hammen, 1944; Hotchkiss, 1945; Hinglais and Hinglais, 1947; Farris, 1950; Lane-Roberts, Sharman, Walker, Wiesner and Barton, 1948; Bayle and Gouygou, 1953; Longo, 1953; Williams, 1953), while others deal with various animals (Gunn, 1936; Burrows and Quinn, 1939; Anderson, 1945; Bonadonna, 1945; Perry, 1945; Walton, 1945; Milovanov and Sokolovskaya, 1947; Van Drimmelen, 1951; Millar and Ras, 1952). In addition, much valuable information on sperm physiology in general, indispensable alike to those engaged in human and in animal research, will be found in the writings of Marshall (1922), Hartman (1939), Chang and Pincus (1951) and Walton (1954), as well as in the published records of various symposia and conferences held under the auspices of such bodies as the Biochemical Society (*Biochemistry of Fertilization and the Gametes*, 1951), the New York Academy of Sciences (*Biology of the Testes*, 1952), the Ciba Foundation (*Mammalian Germ Cells*, 1953), the National

Committee on Maternal Health (*Diagnosis in Sterility*, 1946; *The Problem of Fertility*, 1946; *Studies on Testis and Ovary, Eggs and Sperm*, 1952), the American Society for the Study of Sterility (official journal: *Fertility and Sterility*) and the British Society for the Study of Fertility (*Proceedings*).

SPERMATOZOA

Spermatogenesis and sperm 'ripening'

Spermatozoa (Plate I and Fig. 1) originate in the testis from the germ or spermatogenic cells of the seminiferous epithelium in the

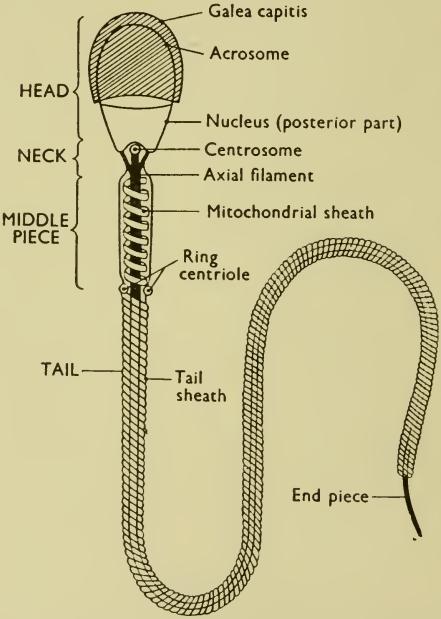


FIG. 1. *Diagrammatic representation of a spermatozoon.*

course of spermatogenesis, a process of stepwise proliferation and transformation, distinguished by the successive stages of spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids. The present knowledge concerning the chemical changes

which take place during spermatogenesis is defective and rests almost entirely on histochemical observations.

In several species so far investigated, spermatogonia and spermatocytes have been shown to have a cytoplasm which is basophilic, in distinction to mature spermatozoa which exhibit only a faint coloration of the flagellum. Cytochemical studies carried out by Brachet (1944, 1947) have shown that the affinity of the spermatogonia and spermatocytes for basic dyes is due to ribonucleic acid, and cytochemical as well as spectrophotometric studies (Caspersson, 1939) point to the fact that spermatogenesis involves a progressive disappearance of ribonucleic acid from the developing sperm cell. In ejaculated spermatozoa of the bull, Vendrely and Vendrely (1948) using the analytical methods of Schmidt and Thannhauser (1945) and Schneider (1945), found a content of $0{\cdot}2\times10^{-9}$ mg. ribonucleic acid per sperm cell, that is fifteen times less than the corresponding value for deoxyribonucleic acid. An analysis of mature ram spermatozoa carried out in our laboratory with the Markham-Smith chromatographic procedure (1949) which is based on the identification of uridylic acid in an acid hydrolysate of ribonucleic acid, failed to reveal the presence of uridylic acid. As to the origin of ribonucleic acid in the spermatogonia and spermatocytes, a study of the spermatogenesis in *Asellus aquaticus* (Vitagliano and de Nicola, 1948) suggests that ribonucleic acid is not elaborated in the developing gametes themselves but is secreted by the surrounding cells and then absorbed and utilized by the germ cells.

Two other processes associated with spermatocytic development are: the progressive decline of alkaline and acid phosphatase activity (assessed histochemically) in the nuclei (Krugelis, 1942; Wolf, Kabat and Newman, 1943), and a simultaneous disappearance of glycogen. Both the Sertoli cells and the spermatogonia abound in glycogen, which also occurs, although in a smaller concentration, in the primary spermatocytes (Montagna and Hamilton, 1951; Elftman, 1952; Long and Engle, 1952; Mancini, Nolazco and Balze, 1952). But the secondary spermatocytes and the spermatids give practically no cytochemical reactions for glycogen, and in the mature spermatozoa the glycogen content is exceedingly low: in ejaculated ram semen 'glycogen' content, i.e. the alkali-resistant polysaccharide which behaves like glycogen on ethanol-precipitation and which

yields on hydrolysis glucose (as determined by glucose oxidase) seldom exceeds 0·1%, and may be as little as 0·019% (Mann, 1946*b*). Similarly, in sea-urchin sperm (*Echinus esculentus*), the ethanol-precipitable, glycogen-like material separated from sperm and analysed after acid hydrolysis by means of glucose oxidase, represents no more than 0·04% on a wet-weight basis (Rothschild and Mann, 1950). Even oysters, in which as much as one-third of the dry body weight may consist of glycogen, produce spermatozoa which when ripe, contain no more than 1% glycogen on a dry-weight basis (Humphrey, 1950).

Yet another phenomenon accompanying spermatogenesis is a significant change in the distribution of lipids. In the deer (Wislocki, 1949) and in the rat (Lynch and Scott, 1951), sudanophilic material is concentrated chiefly in the Sertoli cells, but in man (Montagna, 1952) a high content of lipids is characteristic alike of the Sertoli cells as well as the cytoplasm of spermatogonia and of primary spermatocytes. Similarly, in certain invertebrates, as for example, *Lithobius forficatus* (Monné, 1942), lipids form a highly characteristic component of the cytoplasm in spermatocytes. These cytoplasmic lipids are usually birefringent and give positive reactions for steroids. In ejaculated spermatozoa, at any rate those of mammals, the lipids are confined largely to certain definite regions such as the 'mitochondrial sheath' of the middle-piece and the so-called lipid capsule; these will be described in more detail later.

The changes initiated by spermatogenesis continue during the stay of spermatozoa in the epididymis, and form a part of the 'ripening' process. The metabolism of epididymal spermatozoa which are often immotile, but capable of long survival, is as yet only poorly understood. Guinea-pigs and rabbits, for example, can remain fertile for some weeks after the ligation of the ductuli efferentes, and in bats spermatozoa have been detected in the cauda epididymis as late as seven months after the cessation of spermatogenesis.

A striking change associated with the process of sperm ripening in the epididymis is the migration of a drop-like swelling of sperm cytoplasm called the 'kinoplasmic droplet' and believed to contain some lipid material; when one examines spermatozoa from the caput epididymidis of a mouse for example, the kinoplasmic droplet is usually situated close to the proximal (anterior) end of the middle-

piece, but by the time the spermatozoa have reached the cauda epididymidis and are nearing the vas deferens, the droplets take up a position at the distal (posterior) end of the middle-piece (Merton, 1939; Fig. 2). Finally, they tend to disappear altogether and are seldom found in ejaculated sperm, except in certain abnormal cases (Plate I). Some authors regard the kinoplasmic droplet as no more than a remnant of spermatid cytoplasm devoid of special significance, but there are those who believe that it plays an important role by nourishing the spermatozoon during the passage through the epididymis, before the sperm cells establish contact with an extracellular source of nutrient material, in the form of seminal plasma.

The disappearance of the kinoplasmic droplet is but the final stage in the process of gradual shrinkage and 'dehydration' of protoplasm which accompanies both spermatogenesis and ripening, and from which the 'ripe' spermatozoon ultimately emerges as a cell with a highly condensed nucleus and very little cytoplasm. Associated with the diminution of protoplasm is a progressive loss of water and a corresponding increase in the specific gravity of the sperm cell. Lindahl and Kihlström (1952) suspended equal numbers of bull spermatozoa in a series of aqueous solutions of the methylglucamine salt of 'umbradil' (2 : 5-di-iodine-4-pyridone-*N*-acetic acid), the lightest of which (sp. g. 1·0918, osmotic pressure 18 atm.) had a lower specific gravity than any of the spermatozoa, the heaviest (sp. g. 1·3519, osmotic pressure 220 atm.) being of about the same specific gravity as the 'densest' spermatozoa; these sperm suspensions were centrifuged in haematocrit tubes so that all spermatozoa with a specific gravity exceeding that of the medium, formed a sediment in the graded capillary part of haematocrit tubes. The specific gravity of bull

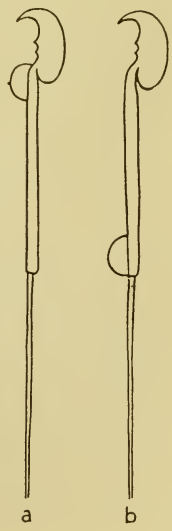


FIG. 2. '*Ripening*' *process in the epididymis of the mouse;* (*a*) spermatozoon from the caput epididymis with proximal kinoplasmic droplet; (*b*) spermatozoon from the cauda epididymis with distal kinoplasmic droplet.

(Merton, 1939)

spermatozoa determined in this manner ranged from 1·240 to 1·334; there was a negative correlation between the mean specific gravity and the percentage of unripe spermatozoa, that is those which still possessed the kinoplasmic droplet; in each experiment, the concentration of unripe spermatozoa was significantly higher in the 'floating', than in the sedimenting, fraction, the specific gravity of the unripe sperm cells being less than that of the ripe ones.

To some extent, the specific gravity of spermatozoa may be accounted for by the high concentration of deoxyribonucleoprotein in the sperm nucleus, but in a large measure it is also due to the state of 'dehydration' which is characteristic of the sperm protoplasm and its protein constituents. Hand in hand with the high specific gravity goes a remarkably high refractive index and light-reflection power of the spermatozoa. In general, the refractive index of most living animal cells lies between 1·350 and 1·367, corresponding to a 10–20% concentration of solids; but in human spermatozoa examined by the immersion method, Barer, Ross and Tkaczyk (1953) obtained values corresponding to a content of almost 50% solids. Nephelometric measurements of light reflection carried out with bull semen samples containing a varying percentage of 'unripe' spermatozoa, showed that the capacity of the sperm cell to reflect light increases with ripening (Lindahl, Kihlström and Ström, 1952); there appears to be a close relationship between the light-reflecting power of sperm and the characteristic 'luminosity' of the surface of spermatozoa under dark-field illumination, which, in all probability, is due to the 'waterlessness' of the lipid capsule surrounding the ripe sperm cell.

Sperm transport in the female reproductive tract and 'capacitation'

There is evidence that the process of sperm ripening is not halted at ejaculation but proceeds in the female reproductive tract, where the sperm cell undergoes a definite change, called capacitation, before it becomes capable of penetrating the egg surface (Austin, 1951; Chang, 1951; Austin and Braden, 1952; Thibault, 1952). It is quite likely that the success which some early investigators had in achieving fertilization with artificially inseminated epididymal spermatozoa, was due to the continuation of sperm ripening processes in the female reproductive tract.

Whether the semen is ejaculated into the uterus (sow), or into the cervix or vagina (cow, rabbit), a certain time is always required for the passage of spermatozoa to the oviducts and for their accumulation in adequate numbers at the site of fertilization. The time needed for some of the spermatozoa at any rate, to arrive at their goal may be relatively short; a quarter of an hour or less, in the rat (Blandau and Money, 1944), cow (VanDemark and Moeller, 1951) and ewe (Starke, 1949; Dauzier and Wintenberger, 1952); a matter of a few minutes in the rabbit (Lutwak-Mann, unpublished). This indicates that in these animals the spermatozoa are conveyed to their final destination thanks to certain concomitant movements of the female tract and do not depend exclusively upon their own motility. However, from the moment of arrival in the ovarian tube, time must elapse before the sperm cell is capable of fertilizing the egg. In the rabbit, ovulation takes place about ten hours after copulation, and presumably, spermatozoa require this period of time to undergo complete 'capacitation'. As Chang (1951) has shown, rabbit spermatozoa placed in the Fallopian tubes soon after ovulation, penetrate a larger proportion of eggs if they had been previously kept for about five hours in the uterus of another doe. According to Austin (1951), rat spermatozoa injected into the periovarian sac of the rat after ovulation, do not begin to enter the eggs until some five hours later. The processes of sperm maturation and capacitation are linked in some as yet not fully understood manner, with the survival of sperm in the female tract. In higher mammals this period is usually limited to one or two days, but the 'longevity' of bird sperm is remarkable, and in bats and the terrestrial isopode *Armadillidium vulgare* the spermatozoa are said to survive in the female tract for many months, in certain insects even for years. In insects, however, this striking behaviour of spermatozoa is probably bound up closely with certain other peculiarities of sperm transport: in many instances, the spermatozoa are conveyed to the female not in a free fluid medium, but are enclosed in a sac or 'spermatophore' which is deposited in the 'bursa copulatrix' or in the vagina; from there, after the sac has been emptied, they move on to the 'spermatheca', a pouch which serves as a special storage organ for the spermatozoa, where they remain till the time of fertilization.

It must also be remembered that not all spermatozoa present in

a given ejaculate survive for the same length of time. In higher mammals for instance, of the many hundreds of millions of sperm cells, only a minute fraction, not more than a few thousand, reach the site of fertilization, and ultimately only a single spermatozoon is responsible for the fertilization of the ovum.

Structural and chemical characteristics of the sperm-head, middle-piece, and tail

In the majority of species, including man, mature spermatozoa have a filiform structure owing to the presence of a flagellate appendage, although non-flagellar forms of sperm cells are not uncommon in certain lower animals, for example, among crustacea and nematodes. This peculiar filiform structure determines to a considerable extent, the remarkable permeability of the sperm cell, which is perhaps best illustrated by the so-called 'leakage' phenomenon, that is, the remarkable ease with which even large molecules such as cytochrome *c* or hyaluronidase can detach themselves from the sperm structure and pass into the extracellular environment. The high degree of permeability explains the speed with which exchange reactions can take place between the spermatozoa and the surrounding medium, whether this be the seminal plasma or an artificial pabulum; moreover it makes it possible for certain intermediary enzymic reactions such as those involved in the phosphorylative breakdown of carbohydrate, to be demonstrated directly in intact spermatozoa, without cell disintegration which is an unavoidable prerequisite in studies on the intermediary enzymes of other animal tissues. This does not necessarily apply to all enzymes and the failure to demonstrate an enzyme in intact sperm cells must not be taken as evidence of its absence, particularly so in the case of mammalian spermatozoa which are resistant to the action of most plasmolysing agents, including water.

The principal morphological features of spermatozoa have been established largely in the last century with the help of the ordinary light microscope, by pioneers such as Ballowitz, Jensen, Meves, Retzius and others, but many more details have emerged since as a result of the application of new techniques, particularly those of *histochemistry* (Marza, 1930; Popa and Marza, 1931; Brachet, 1944; Leblond, Clermont and Cimon, 1950; Leuchtenberger and

a



b



PHOTOMICROGRAPHS

a. Normal bull semen; photographed in ultraviolet light at 2750 A. Mag. × 2700.

b. Semen from an infertile bull; the spermatozoa are 'unripe' and show kinoplasmic droplets at the anterior ends of the middle-pieces; nigrosin-eosin stain.



c



d



e

OF SPERMATOZOA

c. Rat spermatozoon.

d. Rat spermatozoon with a kinoplasmic droplet at the posterior end of the middle-piece.

e. Rat spermatozoon. Mag. × 1500.

(By courtesy of Dr. C. R. Austin and Dr. J. L. Hancock)

Schrader, 1950; Wislocki, 1950; Friedlaender and Fraser, 1952; Hancock, 1952; Melampy, Cavazos and Porter, 1952) and *electron microscopy* (Seymour and Benmosche, 1941; Harvey and Anderson, 1943; Schmitt, 1944; Bretschneider and Iterson, 1947; Bretschneider, 1949*a*, *b*; Grigg and Hodge, 1949; Hodge, 1949; Randall and Friedlaender, 1950; Bayle and Bessis, 1951; Friedlaender, 1952; Challice, 1953; Bradfield, 1954).

In a typical flagellar spermatozoon (Plate I and Fig. 1) it is usually possible to distinguish three regions, viz. sperm-head, middle-piece and tail, but even among closely related species, one encounters an extraordinary diversity of form, size and structure. Moreover, on examining the semen from a single individual, one often finds in addition to the normally shaped spermatozoa, a variety of 'degenerate', 'abnormal' or 'immature' forms which represent every conceivable deviation from the normal cell structure, from 'tapering' and 'double' cells with a double head or tail, to 'giant' and 'monster' cells containing several nuclei and several tails in a mass of cytoplasm. Although a high degree of sperm abnormality is undoubtedly associated with subfertility, normal semen is seldom completely uniform, and human semen for example, is reckoned to contain as a rule, at least 20% of abnormal forms (Pollak and Joël, 1939; Harvey and Jackson, 1945; Hotchkiss, 1945; Lane-Roberts *et al.*, 1948; Williams, 1950). In the bull (Williams and Savage, 1927; Lagerlöf, 1934; Bishop, Campbell, Hancock and Walton, 1954) and stallion (Bielański, 1951), the percentage of abnormal forms in semen is similarly high, in the ram on the other hand, it appears to be much less.

The shape of the head in a normal spermatozoon varies greatly; it is ovoid in the bull, ram, boar, and rabbit, it resembles an elongated cylinder in fowl and has the form of a hook in the mouse and rat; in the human species, the sperm-head appears as a flattened, oval body, about 4·6 μ long, 2·6 μ wide, and 1·5 μ thick, which is compressed at the anterior pole into a thin edge.

The main part of the head is occupied by the nucleus, filled by closely-packed chromatin which consists largely of deoxyribonucleoprotein and gives a positive Feulgen (nucleal) reaction with Schiff's fuchsin-sulphurous acid reagent. The anterior part of the nucleus is covered by a cap-like structure known as the acrosome. The

latter gives no positive Feulgen reaction but stains with the Schiff reagent after exposure to the oxidizing action of periodic acid as demonstrated in the sperm of the hemipteran insect, *Arvelius albopunctatus* (Leuchtenberger and Schrader, 1950) and in bull sperm (Hancock, 1952). According to McManus (1946) and Hotchkiss (1948), the 'periodic acid Schiff reaction' (PAS) is due to the presence of carbohydrates, and the chemical groups which react with fuchsin-sulphurous acid are the aldehydes formed from 1 : 2 glycol groupings by oxidation with periodic acid:

$$\begin{array}{c} \text{OH}\quad\text{OH} \\ |\quad\quad| \\ \text{R—C——C—R} \end{array} + \text{HIO}_4 \longrightarrow 2\text{R—CHO}$$

The acrosomal material is not glycogen as it does not react with iodine and is not affected by treatment with amylase. It cannot be hyaluronic acid because it resists the action of hyaluronidase. The possibility that it may be related to hyaluronidase itself still remains to be investigated. There has also been a tendency to regard it as a mucopolysaccharide, without however, sufficient evidence. Special precautions are called for in the preparation of spermatozoa for the PAS reaction. Structural changes in sperm cells, such as occur for example, after rapid cooling ('temperature shock'), may render the acrosomal material unresponsive to the periodic acid-Schiff reagent. It is not improbable that the acrosomal 'polysaccharide' is either decomposed or detached from the head of a mature spermatozoon; this is borne out by some microscopic observations on changes which take place in the acrosome during the period of senescence and death of the sperm cell. Several investigators have described in spermatozoa yet another cap, a loose protoplasmic structure, named 'galea capitis' (also 'acrosome cap', 'Kopfkappe' or 'capuchon céphalique') which envelops the apical part of the sperm-head and can break away spontaneously to form a so-called 'spermatic veil' or 'floating cap' (Williams and Savage, 1925; Blom, 1945). However, whereas most authors including Williams (1950) regard the acrosome proper and the galea capitis as two distinct structural entities, some consider them to be identical, and Hancock (1952) for instance, is convinced that there is only one acrosomal structure and, that the detachable cap arises through post-mortem changes, and is the result of swelling and loosening of the acrosome itself. The separation of

the galea capitis can be conveniently followed by the India-ink staining technique of Blom (Fig. 3) whose studies indicate that the phenomenon occurs most frequently in degenerating spermatozoa, for example, in ejaculates obtained after long periods of abstinence. When stained by the Gomori technique, the galea capitis or at least the region near the tip of the head, shows a positive reaction for acid phosphatase. On the whole, however, phosphatase activity, 'acid' and 'alkaline' alike, is much more intense in the seminal plasma than in the spermatozoa. Moreover, as it is rather difficult to

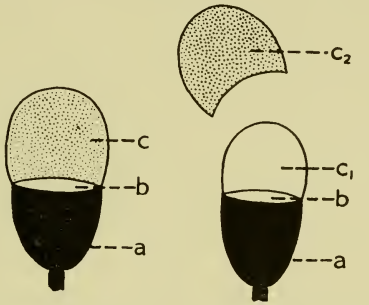


FIG. 3. *Schematic representation of the head of a bull spermatozoon before and after detachment of the galea capitis (India-ink method).*

a, pars posterior, *b*, pars intermedia, *c*, pars anterior with the galea capitis in situ, c_1, the 'bare' pars anterior after detachment of the galea, c_2, galea capitis detached from the head.

(Blom, 1945)

remove from the spermatozoa, even by exhaustive washing, all adhering traces of seminal plasma, the possibility of contamination with plasma phosphatase must be taken into account when considering the occurrence of phosphatase in the spermatozoa themselves.

The narrow region which connects the sperm-head with the middle-piece is known as the neck (or neck-piece), which is the most vulnerable and fragile part of the spermatozoon. The entire neck region is bounded, however, by a membrane which continues over the head and middle-piece. In the neck, close to the base of the sperm nucleus, is situated the centrosome which marks the beginning of the 'axial filament', the central core of both the middle-piece and tail. The axial filament consists of a number of fine long fibrils which

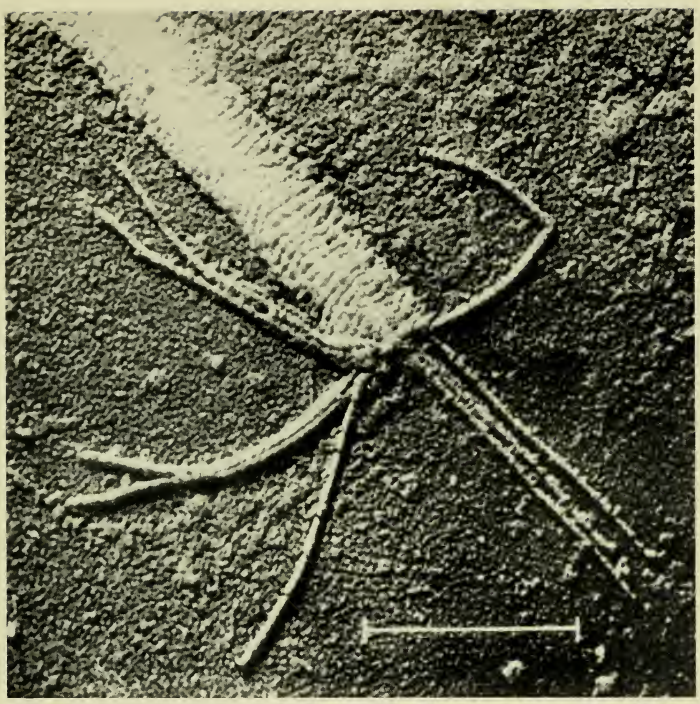
run uninterruptedly through the whole length of the middle-piece and tail. These fibrils probably represent the main contractile element of the sperm cell, responsible for the whip-like lashing of the tail. In most species investigated so far by means of the electron microscope, eleven fibrils have been identified; two of these, which occupy the central position, are sensitive to the action of water and digestive proteolytic enzymes, whereas the remaining nine fibrils which form an 'outer cylinder' around the 'central pair', are remarkably resistant to the action of plasmolysing and digestive agents, and even prolonged proteolysis with pepsin or trypsin fails to disrupt them; these fibrils also resist effectively attempts at solubilization by means of salt solutions, acids and weak bases.

The finer structure of the individual fibrils is still a matter of active investigation. In the case of mammalian spermatozoa, doubling of fibrils has been observed, at any rate in the middle-piece, and in addition to the outer cylinder of nine fibrils, another, so-called inner cylinder has been described, consisting of nine, much thinner fibrils. The precise chemical nature of the fibrillar protein is unknown. A certain resemblance to muscular contraction prompted Engelhardt (1946) to ascribe to the contractile substance of spermatozoa myosin-like properties, and to sperm adenosine-triphosphatase the role of 'spermosin'. However, this claim remains at present unsubstantiated since it was not accompanied by satisfactory evidence that the spermatozoa used for the experiments, were really free from phosphatases, especially the powerful adenosine-triphosphatase, of seminal plasma.

In the middle-piece (or midpiece) which in the human spermatozoon is about the length of the sperm-head though only one-tenth as wide, the axial filament is surrounded by the 'broad helix', also called 'spiral body' or 'mitochondrial sheath'. This lipid-rich structure, which is believed to be derived from mitochondria, has the shape of a broad paired thread, wound helicoidally round the 'outer cylinder' of sperm fibrils. It is here that the cytochrome–cytochrome oxidase system of spermatozoa is believed to be concentrated. The junction between the middle-piece and tail is marked by the presence of a ring centriole.

The tail or 'flagellum' in the human spermatozoon is about ten times the length of the middle-piece and lacks the 'broad helix'

PLATE II



ELECTRON MICROGRAPH OF SPERM-TAIL

Broken end of tail from a bull spermatozoon, showing the tuft of fibrils of the axial filament, and the helical structure of the tail sheath; |———| indicates 1 μ.

(By courtesy of Prof. L. H. Bretschneider and Dr. Woutera van Iterson)

but has instead the much thinner 'tail sheath' or 'cortical helix' which terminates a short distance before the end of the tail, exposing the terminal portion of the axial filament, that is the end-piece. In mammalian spermatozoa, the tail sheath appears as a helicoidally wound cord; when the tail of the spermatozoon is broken, one can see, protruding from the cortical sheath, the brush-like fibrils of the axial filament, and at this point it is also possible to distinguish the helical structure of the tail sheath (Plate II). In fowl spermatozoa on the other hand, there is no evidence of a 'cortical helix', and the axial filament is encased in an amorphous sheath which is easily disrupted by distilled water, causing the axial filament to fray into fibrils. In addition to the various fibrous cortical systems, the sperm cell of many species, including man and the higher mammals, is protected externally by a lipid layer or capsule ('manteau lipidique') evident especially around the tail, and composed of a layer of liproprotein.

SEMINAL PLASMA

Seminal plasma, the extracellular fluid which provides the medium and vehicle for spermatozoa, originates in the accessory organs of reproduction and varies in composition according to species. In lower animals it may be so scarce that the emitted semen takes the form of a very thick lump of spermatozoa, closely packed together. There is little seminal plasma in bird semen and even among some of the mammals, but on the whole, the higher mammals, including man, produce a relatively dilute semen with a considerable proportion of seminal plasma.

Secretory function of male accessory glands

The seminal plasma is a composite mixture of fluids secreted by organs which in the higher species comprise the epididymides, the seminal ducts or vasa deferentia, ampullae, prostate, seminal vesicles (or seminal glands), Cowper's glands and certain other glands located in the wall of the urethral canal. Until a little while ago, the secretory function of the male accessory organs remained obscure chiefly owing to lack of information about the chemical nature of the various secretions. More recently, however, several substances

have been discovered and identified in the accessory secretions, such as citric acid by Scherstén in 1929, prostatic phosphatase by Kutcher and Wolbergs in 1935, fructose by Mann in 1945, phosphorylcholine by Lundquist in 1946, ergothioneine by Leone and Mann, and inositol by Mann in 1951, and glycerylphosphorylcholine by

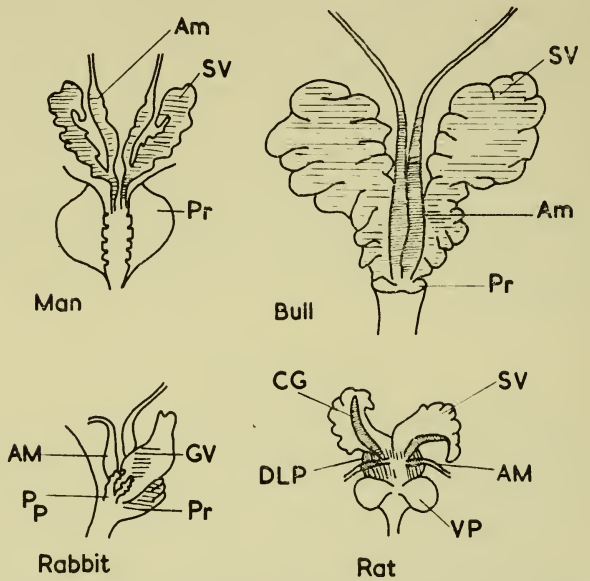


FIG. 4. *Diagrammatic outline of male accessory organs to illustrate the localization of fructose* (*shaded areas*).

Am, ampullae. *SV*, seminal vesicle. *Pr*, prostate. *VP*, ventral prostate. *DLP*, dorsolateral prostate. *Pp*, glandulae paraprostaticae. *GV*, glandula vesicularis. *CG*, coagulating gland.

Diament, Kahane and Lévy in 1952 (for details concerning the secretory function of male accessory organs see: Mann and Lutwak-Mann, 1951*b*; Lutwak-Mann, 1951).

Owing to the complex nature of the seminal plasma the physiologist or biochemist is forced to adopt a distinct approach when investigating any one of the accessory gland secretions. There are several instances where male accessory organs which, though previously believed on the basis of similar embryonic origin or related

PLATE III



REPRODUCTIVE TRACT OF THE BOAR

A, prostate; *B*, seminal vesicle; *C*, vas deferens; *D*, Cowper's gland; *E*, caput epididymidis; *F*, cauda epididymidis; *G*, testis; *H*, bladder. Scale in inches.

morphological structure, to be anatomically and even functionally 'homologous', were later shown to differ greatly in their chemical secretory activity. This is particularly true of the secretions of the prostate and the seminal vesicle, two organs which in the majority of higher species provide the bulk of the seminal plasma; their localization within the reproductive tract of several species is illustrated in Plate III and Fig. 4.

The prostatic secretion

This differs in many ways from other secretions of the mammalian body, and its composition shows considerable species variations. Much study has been devoted to the human and canine prostatic fluids; both are colourless and usually slightly acid, about pH 6·5 (Huggins, 1947; Zagami, 1940) and both are remarkable for the almost complete absence of reducing sugar. They abound, however, in several strong proteolytic enzymes; the human prostatic fluid contains a fibrinolysin so powerful, that 2 ml. of prostatic fluid can liquefy 100 ml. clotted human blood in 18 hr. at 37°; dog prostatic fluid is distinguished by its ability to destroy fibrinogen, but it is relatively inactive towards clotted blood (Huggins and Neal, 1942). The prostate secretes a diastase (Karassik, 1927), and a β-glucuronidase which is more active in man than in dog (Talalay, Fishman and Huggins, 1946; Huggins, 1947).

The prostatic secretion represents the main source of citric acid and of acid phosphatase for whole human semen; and the analysis of these two constituents provides a most convenient 'chemical indicator test' for the assessment of the functional state of the human prostate. There is much more citric acid and acid phosphatase in the human, than in the canine, secretion; thus, the citric acid content is less than 30 mg./100 ml. in dog, as against 480–2680 mg./100 ml. in the human fluid; acid phosphatase activity in dog corresponds to about 28 King-Armstrong units/100 ml. in the 'resting' or spontaneously voided prostatic secretion, and 104 units/100 ml. in the 'stimulated' secretion obtained by parasympathetic stimulation, whereas the prostatic secretion of a normal adult man may contain up to 3950 units/1 ml. (Gutman and Gutman, 1941; Huggins, 1947).

The concentration of osmotically-active substances in the

prostatic fluids of man and dog is shown in Fig. 5. In the human secretion (Huggins, Scott and Heinen, 1942), the average values for cations, expressed in m-equiv./l. water, are: sodium 156, potassium 30, and calcium 30; for anions: citrate 156, chloride 38, bicarbonate 8, and phosphate 1. In a pilocarpine-stimulated dog prostatic fluid (Huggins, Masina, Eichelberger and Wharton, 1939) the base con-

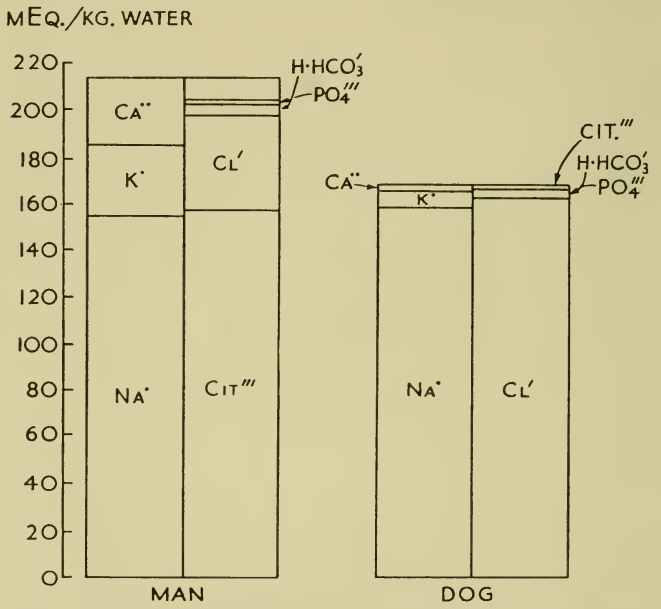


FIG. 5. *Diagram of osmotically active substances in prostatic fluid.* (Huggins, 1947)

sists of sodium 162, and potassium 5; the anions chloride 156, and bicarbonate 1·7 m-equiv./l. In man, the prostatic secretion also provides the main source of calcium; the so-called Niederland reaction, which depends on the formation of characteristic needle-shaped crystals in human semen heated with dilute sulphuric acid, is probably due to calcium sulphate (Niederland, 1931, 1935; Ziemke, 1931).

Among the chemical peculiarities of the prostate gland is its

rather high content of zinc. The first to observe this, and to comment on the possible role of zinc in reproduction, was Gabriel Bertrand; in the two analyses of human prostate carried out by Bertrand and Vladesco (1921) there was found 9·4 and 11·3 mg. Zn per 100 g. fresh tissue, or 49·1 and 53·1 mg. Zn per 100 g. dry weight. More recently, Mawson and Fischer (1951, 1952, 1953) in Canada, found that the mean zinc content of the human prostate gland was 68·2 mg. Zn/100 g. dry wt., which is in considerable excess of zinc content in human liver, muscle, brain, testis or blood. These investigators state that the zinc present in the human seminal plasma is derived chiefly from the prostatic secretion.

A considerable number of studies have been carried out with the rat prostate (Fig. 4). In the rat, there is a distinct anatomical and functional difference between the so-called ventral prostate which secretes only citric acid, and the dorso-lateral prostate which produces both citric acid and fructose (Humphrey and Mann, 1948, 1949). In the dorso-lateral prostate itself, however, it is possible to distinguish three smaller regions, the dorsal or median portion which does not contribute citric acid, and two lateral lobes which are rich in citric acid (Price, Mann and Lutwak-Mann, 1949). It is the dorso-lateral prostate, and more specifically, its two lateral lobes which contain much more zinc than any other soft tissue of the rat, and which at the same time exhibit carbonic anhydrase activity almost equal to that of blood (Mawson and Fischer, 1952); whereas however, the carbonic anhydrase in the rat lateral prostate accounts for no more than one-tenth of the total zinc content, in blood erythrocytes this enzyme is well known to correspond closely to the bulk of the zinc content (Keilin and Mann, 1940).

The protein content of the prostatic secretion is low, less than 1% in man, and a certain proportion of the protein-like material present in the secretory fluid is composed of 'proteoses' which are not precipitated by trichloroacetic acid. Another feature of the prostatic secretion is its elevated content of certain free amino acids, the presence of which is probably the outcome of a combined action of proteolytic and transaminating enzymes in the glandular tissue (Barron and Huggins, 1946*a*; Awapara, 1952*a*, *b*). Human prostatic adenoma contains in 100 g. tissue 50 to 200 mg. glutamic acid in addition to several other amino acids. The average content of amino

acids in protein-free filtrates of ground prostatic adenoma or dog prostate, expressed in terms of millimoles/100 g. tissue, is 34 and 39, respectively. The ventral lobe of the rat prostate contains in a free state nearly all known amino acids, and in addition phosphoryl-ethanolamine, taurine, glutathione, and glutamine. The dorso-lateral lobe on the other hand, in contrast to the ventral prostate, has a much lower content of most amino acids and lacks completely iso-leucine and threonine; it may be added here that it also responds differently to castration and to hormones.

The seminal vesicle secretion (Tables 2 and 3)

In several species, including the rat, guinea-pig and bull, the seminal vesicles alone contribute more fluid than the rest of the

TABLE 2. *Some characteristic constituents of the seminal vesicle secretion in man, bull, boar, rat and guinea-pig*

(In each species, two individuals were examined, and results expressed as averages (mg./100 ml.); the human vesicular secretion may have been contaminated with prostatic fluid. Ascorbic acid was determined as dinitrophenylhydrazone, and the value represents the sum of the oxidized and reduced form.)

	Man	Bull	Boar	Rat	Guinea-pig
Dry weight	1200	7370	15670	975	965
Ascorbic acid	5	14	4	4	9
Citric acid	125	670	560	115	193
Fructose	315	970	52	0*	57
Inorganic phosphorus	10	7	3	15	5
Acid-soluble phosphorus	57	23	32	168	67
Ergothioneine	<1	<1	79	trace	trace

* In rat, fructose is secreted by the coagulating gland, an organ adjacent to the seminal vesicle (see Fig. 4).

accessory glands together. Whereas however, in the rat and guinea-pig the seminal vesicles conform to the pattern of true thin-walled and large 'vesicles', the bull seminal vesicles are more correctly described as seminal 'glands', with multiple lobes of glandular tissue which surrounds a system of ramified secretory ducts. The size and storage capacity of the seminal vesicles, and their secretory output,

are subject to individual variations, which are particularly conspicuous in man. But the storage capacity of the human vesicles is small indeed in comparison with that of the bull or boar. In certain mammals such as the dog or cat, the seminal vesicles are altogether absent. In the rabbit, a combined anatomical and biochemical study of the reproductive system has shown that the two organs known as glandula seminalis and glandula vesicularis, develop from the same diverticulum of the Wolffian duct, and possess a common urethral outlet, so that both these glands together may be regarded as homologous to the seminal vesicles proper of other mammals (Davies and Mann, 1947*a*, *b*; Mann, 1947).

TABLE 3. *Composition of the boar seminal vesicle secretion* (*mg./100 ml.*)

(Analysis of 670 ml. seminal vesicle fluid representing the pooled secretions collected from three boars; sp. g. 1·046; pH 7·2.)

Dry weight	18 635
dialysable	6 565
non-dialysable	12 070
soluble in 66% ethanol	5 347
Ash	463
Chloride	12
Sodium	69
Potassium	215
Calcium	12
Inorganic phosphorus	3
Inorganic sulphur	2
Total nitrogen	1 548
Non-protein nitrogen	50
Ammonia	1
Fructose	57
Glucose	3
Total anthrone-reactive carbohydrate	149
Total aminosugar	81
Inositol	3 053
Total sulphur	342
Ergothioneine	78
Lactic acid	21
Citric acid	560
Total phosphorus	34
Acid-soluble phosphorus	27

Compared with the prostatic fluid, the seminal vesicle secretion is usually less acid, sometimes distinctly alkaline, has a higher dry weight and contains more potassium, bicarbonate, acid-soluble phosphate and protein; the latter is to a large extent precipitable by trichloroacetic acid but there is also some 'proteose' as shown for example, by the study of the seminal vesicle proteins in the goby *Gillichthys mirabilis* (Young and Fox, 1937). But the most remarkable feature of the seminal vesicle secretion is its unusually high content of reducing substances.

The normal seminal vesicle secretion is usually slightly yellowish but occasionally, especially in man and bull, it can be deeply pigmented. The yellow pigmentation is probably of composite origin but much of it is due to flavins which cause the vesicular secretion and seminal plasma to fluoresce strongly in ultraviolet light. Brochart (1952) observed that when strongly yellow coloured samples of bull seminal plasma are exposed to sunlight, the colour tends to disappear within a short time and lumiflavin is formed. Leone (1953) has shown that at least part of the flavin content of the bull seminal vesicle secretion is due to adenine-isoalloxazine dinucleotide, associated with xanthine oxidase. The highest content of total flavin which I was able to record in the bull seminal vesicle secretion, was 750 μg./100 ml.; in eight samples of bull seminal plasma there was from 47 μg./100 ml. (in an almost colourless specimen) to 480 μg./100 ml. flavin (in a particularly deeply pigmented specimen). There can be little doubt that the flavin associated with the strongly yellow-coloured specimens of bull semen is due principally to the seminal plasma, and not to the spermatozoa. The sperm cells themselves, however, contain also some flavin. In washed bull spermatozoa, there is some 30 μg. riboflavin/g. dry weight (Lardy and Phillips, 1941*c*), and in whole bull semen, particularly in the less coloured samples, a substantial portion of flavin may be derived from the spermatozoa (VanDemark and Salisbury, 1944).

In addition to the yellow pigment, the seminal plasma sometimes contains a brownish haematin pigment; this occurs in cases of 'chronic haemospermia', a condition occasionally met with in man and attributed to haemorrhagic changes in the seminal vesicles (McDonald, 1946).

Potassium in a high concentration occurs in the vesicular secretion

of several species, including man (20 mM), bull (100 mM) and boar (300 mM). In the latter, the ionic equilibrium on the cationic side is set up chiefly by potassium, with citric acid as the main anion; the concentration of sodium is much less than that of potassium; chlorides are conspicuous by their almost total absence, a phenomenon infrequently encountered in other normal body fluids (Table 3). Another unusual feature of the boar vesicular secretion is its high content of inositol which varies from 2 to 3% and accounts for something like one-third of the total dialysable material; inositol together with citrate, contributes substantially to the osmotic pressure of the vesicular secretion (Mann, 1954).

The reducing power of the vesicular secretion is one of its most characteristic chemical properties. Two kinds of reducing substances are present. One group is made up of substances which are capable of reducing silver nitrate, iodine and 2 : 6-dichlorophenol indophenol in the cold. They are always present in the protein-free filtrate from the vesicular secretion and seminal plasma but their chemical nature varies from one species to another. At one time, the reducing property was generally attributed to ascorbic acid, in the secretions of the guinea-pig (Zimmet and Sauser-Hall, 1936; Zimmet, 1939), bull (Phillips, Lardy, Heiser and Ruppel, 1940; Jacquet, Cassou, Plessis and Brière, 1950) and man (Nešpor, 1939; Berg, Huggins and Hodges, 1941). According to Phillips and his co-workers, bulls of high fertility produce semen containing 3 to 8 mg. ascorbic acid per 100 ml., whereas low-fertility bulls may have less than 2 mg./100 ml.; these workers have also claimed that in certain bulls it was possible to enhance the fertility by parenteral administration of ascorbic acid. More recent studies, however, based upon chemical methods of purification and identification, have proved that ascorbic acid rarely accounts for the total reducing power of semen towards dichlorophenol indophenol. In the boar especially, ascorbic acid has been shown by Mann and Leone (1953) to account but for a small fraction of seminal reducing power and the bulk of the reducing material was found to consist or ergothioneine, a substance which owes its reducing power to a sulphydryl group (Leone and Mann, 1951; Mann and Leone, 1953). The properties and functions of ergothioneine will be discussed later (p. 174).

The presence of the other kind of reducing substances can be detected in protein-free filtrates from semen and seminal vesicle fluid, by heating with sugar reagents, such as for example, cupric hydroxide. In this category belongs fructose, the physiological sugar of semen.

Huggins and Johnson (1933) were the first to observe that the reducing sugar of human semen is derived from the secretion of the seminal vesicles but is absent in the prostate. Similar findings were made with the bull (Bernstein, 1937), boar (McKenzie, Miller and Bauguess, 1938) and ram (Moore and Mayer, 1941). The identification of the seminal sugar as fructose (Mann, 1946*a*, *b*, *c*) opened the way for detailed studies of the fructose-generating capacity of the accessory tissues (Fig. 4). It was shown that in several species fructose is secreted either by the seminal vesicles or by functionally related organs (Mann, 1946*c*; 1947; 1948*a*, *b*). This made it possible to use the chemical assay of fructose in semen as an indicator of the relative contribution made by the seminal vesicles towards the make-up of the whole semen. It must be pointed out, however, that a certain small amount of fructose is also produced by the ampullar glands and in some species, by certain other accessory organs (see p. 138).

The physiological function of the seminal plasma

From time to time doubt is expressed as to whether the individual accessory gland secretions or even the entire seminal plasma, have any essential role to fulfil in the process of reproduction; the more so, since in some animals such as the guinea-pig or rabbit, it is possible to induce pregnancy by the artificial insemination of epididymal spermatozoa. It is however, arguable as to how much significance may be ascribed to such experiments, and it is certain that the natural mating process could scarcely be expected to function smoothly and efficiently without the provision of seminal plasma as a normal diluent and vehicle for the thick mass of closely packed epididymal spermatozoa; no more could the blood corpuscles act as oxygen carriers *in vivo*, without the blood plasma.

Furthermore, the seminal plasma exerts a distinct stimulating effect on sperm motility. In part, this is due simply to the 'dilution effect', a phenomenon which is described fully elsewhere (see p. 73).

To a considerable extent, however, the activation by seminal plasma has been shown to be linked with the occurrence of specific substances in the different accessory gland secretions. In certain insects, *Bombyx mori* for example, the spermatozoa are believed to acquire their full fertilizing capacity only after activation by the secretion of the lower portion of the male tract, the so-called glandula prostatica (Wigglesworth, 1950). Many investigators have studied the activating influence of the prostatic secretion on mammalian spermatozoa (Steinach, 1894; Hirokawa, 1909; Ivanov, 1929; Sergijewski and Bachromejew, 1930), and in some instances, e.g. the dog, have claimed it to be species-specific (Ivanov and Kassavina, 1946). By no means all of these experiments, however, have been carried out under satisfactorily controlled conditions and, as Huggins (1945) thinks, in many cases it is impossible to exclude the action of non-specific factors such as acceleration of sperm motility by certain ions or by changes in the gas tension.

Much discrimination is equally needed in appraising certain results obtained with the epididymal secretion. Here, however, the problem involves not so much the 'activation', as the 'ripening' and 'life-prolonging', effects on the spermatozoa (Tournade and Merland, 1913; Stigler, 1918; Braus and Redenz, 1924; Hammond and Asdell, 1926; Young, 1929, 1930; Young and Simeone, 1930; Lanz, 1931, 1936; Gunn, 1936; Gunn, Sanders and Granger, 1942). Most investigators agree that in some special way the epididymis is adapted for the long-term storage of spermatozoa; but whereas some like Braus and Redenz (1924), attribute to the epididymal secretion a specific role in the 'ripening' process, others deny it such a function. In the seminal vesicle fluid, we seem to be confronted with an activating effect on spermatozoa different from that exerted by the prostatic and epididymal secretions, one which is specifically bound up with the presence therein of fructose which provides a source of nutrient material for the sperm cells.

Another facet of the physiological function of the seminal plasma concerns certain characteristic pharmacological effects of the accessory gland secretions and the role of seminal plasma in the remarkable process of semen coagulation and liquefaction.

Prostaglandin, vesiglandin, and certain other pharmacodynamically active substances

Among the more striking pharmacological effects of seminal plasma are a depressor action on blood vessels, and a stimulation of isolated smooth-muscle organs such as the uterus and the intestines. Both these effects which have been studied in great detail by von Euler (1934*a*, *b*; 1935, 1937, 1939, 1949) and Goldblatt (1933, 1935*b*) are due in all probability not to a single substance but to the combined action of several constituents of seminal plasma, including choline and two substances which Euler has named 'prostaglandin' and 'vesiglandin'. So far, only prostaglandin has been purified. The purest preparation obtained from ram prostate glands has been found by Bergström (1949) to be nitrogen-free, and to contain an unsaturated acidic substance which absorbs strongly ultraviolet light with a maximum at 280 mμ. When assayed on the isolated intestine of rabbit, 1μg. of this substance exhibited the same activity as the crude extract from 500 mg. prostatic tissue. Assuming that the substance is pure, the total content of prostaglandin in 100 kg. prostate glands must be of the order of 25–50 mg. To prepare this quantity, one would require the glands from 20,000 rams.

The physiological significance of prostaglandin and vesiglandin in reproduction processes is unknown but it has been suggested that they represent some sort of 'automatic regulator' which controls the voiding of the secretions from the prostate and the seminal vesicle, respectively. The idea of chemical stimulation by secretory products is based among others, on observations that the emptying of the prostate and seminal vesicle leads to a decreased ability of these glands to contract which persists until enough of freshly formed secretion has accumulated.

A pharmacodynamic influence of the seminal plasma upon some parts of the female reproductive tract has also been envisaged but the evidence is derived mainly from experiments on isolated organs. It remains questionable whether any of the effects exerted by the seminal plasma on the uterus and oviduct *in vitro*, also occur in the female reproductive tract *in vivo* (Barnes, 1939; Asplund, 1947).

Kurzrok and Lieb (1931) found on adding 1 ml. of human seminal fluid to a strip of human uterus suspended in a 100 ml. bath, either

an increase or a decrease in spontaneous contractions. Cockrill, Miller and Kurzrok (1935) observed that those specimens of human semen which were capable of enhancing uterine contraction, caused an inhibition after having been exposed for half an hour to pH 10; at pH 11 all specimens became inactive. Moreover, the effect was potentiated by eserine and suppressed by atropine. The observed action corresponded to that of about 100 μg. acetylcholine per ml. semen. About that time, the occurrence of a powerful oxytocic substance in the human seminal plasma was demonstrated by Goldblatt (1935*b*) who also found that the activity was destroyed by short boiling of the seminal plasma with either 0·1N-NaOH or 0·1N-HCl. When assayed on the guinea-pig's uterus, 1 ml. of human seminal plasma showed approximately the same oxytocic activity as 0·4–0·6 mg. of histamine. Euler (1937) believes that the oxytocic activity is due to prostaglandin which in his experiments stimulated strips of human uterus and also isolated uterus as well as uterine strips from several species including the cow, rabbit, guinea-pig and rat. Asplund (1947) determined the total content of 'contractive substance' in 155 specimens of human semen which he assayed on the rabbit intestine *in vitro*, with purified prostaglandin as the standard. He came to the conclusion that the effect of semen on the isolated intestine must be attributed to a combined action of prostaglandin, choline, and at least one other substance which produces a very rapid increase in tonus and is unaffected by atropine. There was no correlation between the total content of 'contractive substance' in seminal plasma and the motility and viability of spermatozoa.

In addition to the remarkable vaso-dilation and contraction of plain muscle, the seminal plasma and the accessory gland secretions exhibit a characteristic pressor activity towards blood vessels. In 1906 Jappelli and Scafa found that parenterally administered extracts of the canine prostate produced an increased blood pressure in the dog and stimulated the respiration. A similar effect was observed by Thaon (1907) after the intravenous injection of prostatic extracts into rabbits but in his experiments the rise in blood pressure was usually followed by a fall. There are indications that the pressor action of the prostatic extracts is due to adrenaline-like substances demonstrated in semen and accessory glands by Collip (1929), v. Euler (1934*b*), Bacq and Fischer (1947), and Brochart (1948*a*) (see p. 181).

Coagulation and liquefaction

Semen is ejaculated in a liquid or semi-liquid form. In some animal species such as the bull and the dog, it remains liquid, but in others it tends to gelate or coagulate shortly after ejaculation. Human semen clots immediately after ejaculation only to liquefy again a little later; until that happens the spermatozoa do not become fully motile. For this reason the examination of human spermatozoa should be postponed until at least twenty minutes after the emission of semen (McLeod, 1946*a*; Simmons, 1946). Quite fresh boar semen usually contains only small lumps of gelatinous material somewhat resembling tapioca; on standing, however, the lumps increase and merge into a semi-solid gelatinous mass which may take up half or more of the entire ejaculate. Gelation of semen can also be observed in the stallion. Even more striking is the clotting phenomenon in the rodents. The major part of a rabbit ejaculate collected by means of an artificial vagina often consists of a colourless transparent gel. In rats and guinea-pigs, semen coagulation leads to the formation after mating, of the so-called *bouchon vaginal* or vaginal plug, which probably prevents the back-flow of semen from the vagina and assists the passage of spermatozoa through the cervix into the uterus. In a study of sperm transport in the rat, Blandau (1945) has shown that the ejaculate fails to pass through the uterine cervix if the coagulation of semen is abolished by ligation of the seminal vesicle and coagulating gland ducts. The copulatory plug has also been described in *Insectivora* (mole and hedgehog), *Chiroptera* (*Rhinolophidae* and *Vespertilionidae*) and in *Marsupialia* (Camus and Gley, 1899; Courrier, 1927; Eadie, 1939, 1948*a*, *b*; Engle, 1926; Rollinat and Troussart, 1897; Stockard and Papanicolaou, 1919). In most animals the vaginal plug is due to the clotting of the semen itself but in some, namely in the opossum (Hartman, 1924) and in the bat *Vesperuga noctula* (Courrier, 1925; Grosser, 1903), its occurrence involves the coagulation of female secretions by the seminal plasma. In the honey bee, the escape of semen from the female reproductive tract is prevented by the so-called mucus plug; this is formed by the material ejected from the mucus glands of the drone towards the end of ejaculation (Laidlaw, 1944).

In most species the substrate for gel formation consists of protein-

like material secreted by the seminal vesicles (Bergmann and Leuckart, 1855; Bischoff, 1852; Hensen, 1876; Landwehr, 1880; Lataste, 1888; Pittard, 1852; Stockard and Papanicolaou, 1919), the enzyme however, responsible for the coagulation is absent in the vesicles themselves and comes into contact with the protein substrate only in the course of ejaculation. The first to recognize the enzymic nature of the coagulating agent were Camus and Gley (1896, 1899, 1907, 1921, 1922) who named it 'vesiculase'. It was shown by Walker (1910) that the source of the coagulating enzyme in small rodents is the so-called 'coagulating gland', which lies adjacent to the seminal vesicle. The clotting power of the secretion from the coagulating gland is such that 1 part is sufficient to clot 20,000 parts of the seminal vesicle secretion. This quantitative relationship can be demonstrated *in vitro* by collecting the two fluids separately and then mixing them after suitable dilution (Moore and Gallagher, 1930).

The liquefaction of human semen which follows its coagulation, is also an enzymic process. Fibrinolysin and fibrinogenase, two proteolytic enzymes of semen (see p. 114), are believed to play a part in this process.

CHAPTER II

Chemical and Physical Properties of Whole Ejaculated Semen

Species and individual variations in the composition of semen. Presperm, sperm-containing, and post-sperm fractions in the ejaculate. Criteria for the rating of semen quality. Optical and electrical properties of semen. Viscosity, specific gravity, osmotic pressure, and ionic equilibrium. Hydrogen ion concentration and buffering capacity. Metabolism of semen and its relation to sperm density and motility; glycolysis; methylene-blue reduction test; respiration.

Species and individual variations in the composition of semen

A CHARACTERISTIC feature of whole semen is the variability of its composition not only among different species but also between individuals of the same species (Table 4). Somewhat less pronounced but still significant are the variations in the concentration of some of the semen constituents in the same individual (Table 5). All this is not altogether unexpected since both the spermatogenic activity of the testes and the secretory function of the male accessory organs are subject to considerable physiological fluctuations of hormonal origin and are influenced by factors such as light, temperature, season, state of nutrition etc. The variability of semen composition is the reason why repeated examination of whole semen, even if restricted to a single experimental subject, and carried out under identical experimental conditions, need not yield the same quantitative results, as might, for example, an analogous series of blood analyses in the same individual. Therefore, an accurate estimate of semen quality in any one individual cannot be formed on the basis of a single analysis and involves several examinations.

In Table 5 are shown individual fructose variations in the semen of man, bull, ram and rabbit. The analytical results are given in terms of *concentration* (mg./100 ml. semen) and of *absolute content*

(mg./ejaculate). This method of expressing the findings of semen analysis is very useful particularly in the evaluation of pathological abnormalities in the composition of semen. The data in Table 5, especially those relating to rabbit semen, show that the variations in the absolute content of fructose per single ejaculate are much less than those in concentration; the reason being that a rabbit

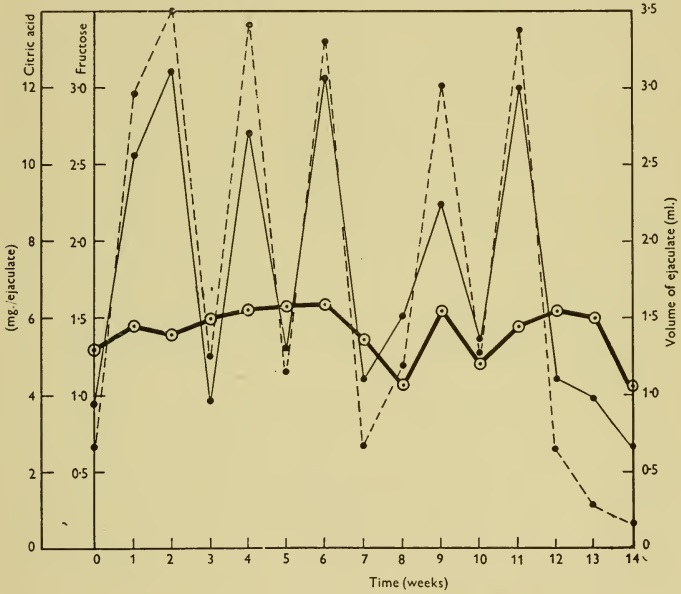


FIG. 6. *Relation between volume* (●————●) *of ejaculate and content of fructose* (⊙————⊙) *and citric acid* (●- - - -●) *in rabbit semen*

(Mann & Parsons, 1950)

ejaculate is usually made up of two main portions, gel and fluid, of which the latter is relatively constant whereas the quantity of gel is subject to considerable fluctuation. Fructose, however, is confined chiefly to the fluid portion and consequently, its *absolute* content in semen varies little. On the other hand, citric acid, another

TABLE 4. *Species differences in the chemical composition of semen*

(Results are average values (range in brackets) expressed in mg./100 ml. except for CO_2 content (ml./100 ml.); for information on the composition of dog and cock semen see appendices (6) and (7).)

	Man[1]	Bull[2]	Ram[3]	Boar[4]	Stallion[5]
Dry weight	8200 (5600–10900)	9530	14820	4600 (2200–6200)	2450
Chloride (Cl)	155 (100–203)	371 (309–433)	87	328 (258–428)	264 (86–443)
Sodium	281 (240–319)	109 (57–201)	103	646 (280–837)	68
Potassium	89 (66–107)	288 (150–415)	71	243 (83–382)	62
Calcium	25 (21–28)	34 (24–45)	9	5 (2–6)	20
Magnesium	14	12	3	11 (5–14)	3
Inorganic phosphorus	11	9	12	2	17
Total nitrogen	913 (560–1225)	756	875	613 (334–765)	167
Non-protein nitrogen	75 (53–107)	48	57	22	55
Urea	72	4	44	5	3
Uric acid	6	6	6	3	
Ammonia	2	2	2	1	1
Fructose	224 (91–520)	540 (280–770)	247	12 (5–25)	15 (9–45)
Lactic acid	35 (20–50)	29 (15–43)	36	27	15
Citric acid	376 (96–1430)	720 (340–1150)	137	141 (41–325)	50 (30–110)
Total phosphorus	112	82	357	66	19
Acid-soluble phosphorus	57 (27·5–93·5)	33	171	24	
Lipid phosphorus	6	9	29	6	
CO_2-content	41–60	16	16	50	24

(1) *Human semen.* Data on dry weight, total nitrogen, and electrolytes (excluding inorganic phosphate): Huggins, Scott and Heinen (1942); CO_2: Sheldovsky *et al.* (1940); urea: Goldblatt (1935*a*); Lactic acid: Lundquist (1949*b*). Remaining data are our own; the results on non-protein nitrogen, fructose, and citric acid content are based on analysis of semen from ten individuals. The ash content of human semen is about 0·9% (Slovtzov, 1902, 1916); the ash contains 3% sulphur (Albu, 1908). According to Infantellina (1945) human semen contains 30 mg./100 ml. glutathione. The values reported for ascorbic acid are 12·8 mg./100 ml. (Berg, Huggins and Hodges, 1941) and 2·6–3·4 mg./100 ml. (Nešpor, 1939); using Roe's method, we found 10·2 and 12·4 mg./100 ml. in two specimens.

(2) *Bull semen.* Data on electrolytes (except magnesium and inorganic phosphate): Bernstein (1933); CO_2: Shergin (1935); remaining data are our own, based on analysis of ejaculates from ten bulls (average volume of ejaculate 4·5 ml.; sperm concentration 985,000/μl.). Other constituents of bull semen include (mg./100 ml.): thiamine 0·028–0·152, riboflavin 0·152–0·306, pantothenic acid 0·230–0·466, and niacin 0·248–0·554 (VanDemark and Salisbury, 1944). Ehlers *et al.* (1953) reported the following mean values based on the analysis of 663 samples of semen (mg./100 ml.): fructose 552 (S.D. 169), citric acid 724 (S.D. 192). For additional data see Rothschild and Barnes (1954).

(3) *Ram semen.* With the exception of the CO_2 content (Shergin, 1935), the data are our own, based on analysis of material pooled from ten ejaculates (average volume of single ejaculate 1·2 ml.; 2,940,000 sperm/μl.).

(4) *Boar semen.* Data on dry weight, electrolytes and total nitrogen: McKenzie, Miller and Bauguess (1938); remaining data our own. Additional information in Table 7.

(5) *Stallion semen.* Data on dry weight, electrolytes, total phosphorus and CO_2 content from Slovtzov (1916), Bernstein (1933), Shergin (1935) and Milovanov (1938); remaining data our own.

(6) *Dog semen.* Much work has been done on the dog prostatic secretion (see text). Whole semen has been analysed by Slovtzov (1916) and the following composition found (mg./100 ml.): dry weight 2450, albumin, globulin and nucleoprotein 866, mucoprotein 57, and lipid 182. Electrolytes have been examined by Bernstein (1933); dog semen is distinguished by a high content of chloride (620–657 mg./100 ml.). Fructose and citric acid occur only in traces.

(7) *Cock semen.* In material from six pooled ejaculates, 5,100,000 sperm/μl., we found (mg./100 ml.): 57 total anthrone-reactive carbohydrate, of which 4 was fructose and 41 glucose (determined by glucose oxidase); 44 total phosphorus, of which 27 was acid soluble; 2 ammonia. In six individual specimens of cock semen we found from 7·7 to 81 mg./100 ml. glucose but never more than 4 mg./100 ml. fructose.

TABLE 5. *Individual variations in the level of fructose in semen*

(Ejaculates collected at weekly intervals.)

Individual (no.)	Semen collection (no.)	Man		Bull		Ram		Rabbit	
		(mg./100 ml.)	(mg./ejaculate)	(mg./100 ml.)	(mg./ejaculate)	(mg./100 ml.)	(mg./ejaculate)	(mg./100 ml.)	(mg./ejaculate)
1	1	250	8·9	480	19·2	340	3·2	108	0·55
	2	250	9·1	510	18·1	268	3·3	230	0·60
	3	210	10·9	390	18·0	285	2·7	70	0·49
	4	180	8·1	390	23·0	466	3·8	40	0·62
	5	250	12·8	490	25·4	350	3·1	108	0·61
	6	230	7·9	450	20·0	290	2·1	150	0·42
2	1	430	12·0	680	32·5	188	1·9	320	1·95
	2	400	12·3	580	32·5	274	1·9	295	1·70
	3	280	15·2	450	29·8	310	2·1	310	1·65
	4	390	21·1	510	27·1	195	2·3	70	2·23
	5	290	11·4	750	33·6	250	2·6	290	1·87
	6	430	12·9	800	30·2	345	2·0	50	2·41

characteristic chemical component of rabbit semen, is found largely in the gel, and its varying content reflects the variability of the latter (Fig. 6).

Frequency of ejaculation also affects the composition of individual samples of semen. Table 6 shows the results of a so-called *exhaustion effect* on the semen of a bull (Mann, 1948*a*); in this experiment, eight ejaculates were collected from the same animal within 63 min., at 7–10 min. intervals. As a result of the multiple collections, the sperm density fell from 1,664,000 cells/μl. in the first, to 98,000 cells/μl. in the last, ejaculate. This decrease, however, was not accompanied by a corresponding diminution of fructose concentration, which was much the same in the first (760 mg./100 ml.) and last (690 mg./100 ml.) ejaculate. But it must be remembered that the seminal vesicles of a bull differ from those of man and other mammals by their exceptional capacity: in some bulls it is possible to recover up to 50 ml. of secretory fluid from the seminal vesicles, enough to provide at least a dozen fructose-rich ejaculates.

TABLE 6. *Effect of frequency of collection on sperm density and on concentration of fructose and lactic acid in fresh bull semen* (Mann, 1948*a*)

Ejaculate no.	Time of collection (min.)	Volume of ejaculate (ml.)	Sperm density (thousands/1μl. semen)	Fructose (mg./100 ml. semen)	Lactic acid (mg./100 ml. semen)
1	0	4·2	1664	760	35
2	10	3·9	680	790	23
3	18	3·7	254	900	22
4	28	3·7	648	750	22
5	38	3·4	135	820	23
6	45	3·5	342	820	22
7	55	2·7	390	630	8
8	63	2·9	98	690	29

Pre-sperm, sperm-containing, and post-sperm fractions in the ejaculate

To assess correctly the composition of individual specimens of semen it is essential to bear in mind the fact that in some species (e.g. man, boar, stallion) the different portions of semen follow one another in a definite order of sequence. This has been demonstrated by the so-called *split-ejaculate method* which depends on the collection and analysis of separate fractions of the same ejaculate according to the time of delivery from the urethra. In man, the ejaculation is initiated by the secretion of Cowper's glands, the prostatic secretion is delivered next, to be followed by the sperm and the vesicular secretion (Broesike, 1912; Huggins and Johnson, 1933; MacLeod and Hotchkiss, 1942; Lundquist, 1949*b*; Pryde, 1950); according to Lundquist, in man the prostatic secretion contributes from 13 to 32% and the vesicular secretion from 46 to 80% of the whole ejaculate.

In the boar, an animal with a protracted period of ejaculation, the semen consists of two portions, gel and liquid. McKenzie *et al.* (1938) calculated that 15–20% of the liquid portion is derived from the seminal vesicles, 2–5% from the epididymis, 10–25% from Cowper's gland, and the rest is made up by the urethral glands secretion. We have made a similar investigation (Mann and Glover, 1954) using estimations of sperm concentration in ejaculated and epididymal semen for the assessment of the epididymal contribution, and the chemical determinations of fructose, citric acid and ergothioneine in the ejaculated semen and in the vesicular secretion as

a means for the evaluation of the contribution of the seminal vesicles. From the results of this study (Table 7) it can be seen that in ejaculated semen (liquid portion), the sperm concentration was 3%

TABLE 7. *Composition of ejaculated semen, epididymal semen, and seminal vesicle secretion of the same boar*

(Four ejaculates were collected from the same boar, at weekly intervals. The boar was then killed, and the epididymal semen and vesicular secretion collected. For chemical analysis, the fluid portions were used, separated from sperm and gel by centrifugation. In the case of ejaculated semen, the means (and standard deviation) are given per single ejaculate.)

	Semen as ejaculated	Semen from the epididymis	Seminal vesicle secretion
Total volume (ml.)	375 (±24)	16	284
Sperm concentration (thousand/μl.) in the liquid portion	108 (±9)	3635	0
Volume of fluid portion (ml.)	292 (±12)	13	283
Composition of the fluid portion (mg./100 ml.)			
Fructose	9 (±0·5)	trace	43
Ergothioneine	17 (±1·1)	trace	81
Citric acid	173 (±9)	14	831
Lactic acid	21 (±3)	19	21
Total dry weight	3970	6420	15020
Dry wt. of non-dialysable material	2745	4235	10615
Total phosphorus	39	227	34
Acid-soluble phosphorus	32	224	31
Sodium	587	88	62
Potassium	197	188	212
Calcium	6	5	12
Hexosamine (after acid hydrolysis)		128	80

($\frac{108}{3635}$) of that present in the epididymis, and the concentrations of fructose, ergothioneine and citric acid, were 20·9% ($\frac{9}{43}$), 20·9% ($\frac{17}{81}$), and 20·8% ($\frac{173}{831}$), respectively, of those present in the seminal vesicle secretion. In semen collected from the boar by the 'split-ejaculate method' it is possible to distinguish clearly three distinct fractions. According to McKenzie *et al.* (1938), the pre-sperm

fraction comprises 5–20%, the sperm-rich fraction 30–50%, and the post-sperm fraction 40–60% of the total ejaculate. The pre-sperm fraction which is ejaculated first, consists of a watery, often discoloured, more or less sperm-free secretion, probably of urethral

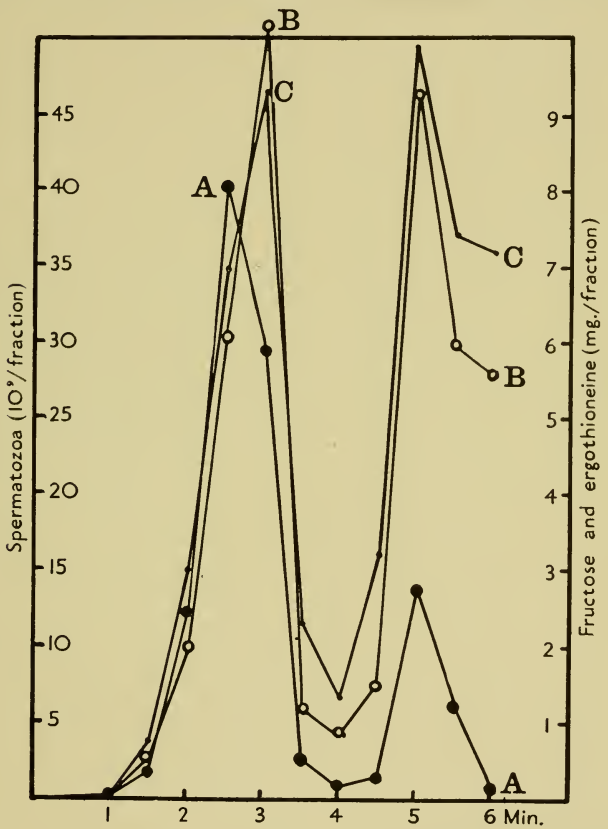


FIG. 7. *Composition of boar semen fractions collected by the 'split-ejaculate method' at half-a-minute intervals.*

A, sperm number $\times 10^9$/fraction (●————●); *B*, fructose content, mg./fraction (○————○); *C*, ergothioneine content, m./fraction (•————•).

(Glover & Mann, 1954)

origin. Within a few minutes, that is followed by a sperm-containing fraction which may also contain some gel-like material. As a rule however, the gel forms a distinct fraction and is voided shortly after the sperm fraction. Moreover, these three fractions, which complete an ejaculation 'wave', may be succeeded by a second 'wave', also fractionated. The two waves together may last up to 30 min., and represent in fact, two successive ejaculates. By the estimation of sperm concentration coupled with the chemical determination of fructose, citric acid, and ergothioneine in the various fractions, it is possible to show that during the ejaculation the seminal vesicle secretion follows immediately upon the delivery of the spermatozoa, and is found mainly in the sperm-containing fraction (Glover and Mann, 1954; Fig. 7). Occasionally, however, the ejaculation of semen is incomplete and the semen does not include the later fractions. When this happens, for example in the stallion, certain normal constituents such as fructose or citric acid, may be missing altogether. Obviously, such incomplete ejaculations create a further complication in the assessment of analytical results obtained with semen, at any rate in those species in which the ejaculation is a fractionated one. But even in the bull where under physiological conditions ejaculation appears to be instantaneous, the 'split-ejaculate method' applied by Lutwak-Mann and Rowson (1953) with the aid of electric stimulation, demonstrated the occurrence in electrically-induced ejaculates, of at least two distinct fractions; of these, the first was a copious sperm-free fraction, slightly viscous, colourless, and of urethral origin, in which there was no fructose or citric acid and very little protein; the next was a creamy sperm-containing fraction, followed by, or more often mixed with, a yellowish-coloured post-sperm fraction which represented an almost pure secretion of the seminal vesicles, with a characteristically high content of fructose, citric acid, and 5-nucleotidase.

Criteria for the rating of semen quality

General medical and veterinary experience indicates that the successful fertilization of the ovum and initiation of pregnancy, while it is brought about primarily by the spermatozoa present in an ejaculate, demands nevertheless the attainment of certain qualitative and quantitative standards by the semen. We should therefore,

be able to define what constitutes normal fertile semen, and what criteria if any, can be applied in the appraisal of ejaculated semen. It may be stated at once that in spite of the wealth of information gained by past and present students of semen, there is as yet no single seminal characteristic known, which alone could serve as the means of judging 'male fertility'. The best criterion of the fertilizing capacity of spermatozoa is of course, the actual ability to fertilize the ovum. This however, cannot be regarded as a laboratory test, until the *in vitro* fertilization of the ovum has actually been accomplished and the quantitative aspects of the process developed.

In the practice of artificial insemination of cattle, male fertility continues to be assessed on the basis of the 'conception rate'. At the artificial insemination centres, inseminated cows which have not been 'returned' by the farmers for re-insemination within three months or so, are presumed to have conceived, and the proportion of presumed pregnancies, expressed as the percentage of the total of the first inseminations, is referred to as 'conception rate'. Nearly one-third of the cow population of England is now bred by artificial insemination, and among these the conception rate averages at least 60%. There is considerable evidence, however, that a substantial proportion of cows 'returned' for re-insemination, may have also conceived but that pregnancy terminated at an early stage through faulty ovum implantation or embryonic death.

Apart from the test based upon accomplished fertilization, the means available at present for the evaluation of semen quality include the histological and the physico-chemical methods.

Histological examination of semen involves procedures such as the determination of sperm concentration or 'density' (number of spermatozoa per 1μl. or 1 ml. of semen) with a cytometer (Walton, 1927; Weisman, 1942); differential count of abnormal forms of spermatozoa (Lagerlöf, 1934; Harvey and Jackson, 1945; Lane Roberts *et al.*, 1948; Williams, 1950); bacteriological examination (Gunsalus, Salisbury and Willett, 1941; Kelly, 1947; Foote and Salisbury, 1948; Almquist, Prince and Reid, 1949; Wu, Elliker and McKenzie, 1952–3); determination of the incidence of dead spermatozoa by means of 'live-dead staining' methods (Lasley, Easley and McKenzie, 1942; Lasley and Bogart, 1943; Madden, Herman and Berousek, 1947; Crooke and Mandl, 1949; Blom, 1950; Mayer,

Squiers, Bogart and Oloufa, 1951; Ortavant, Dupont, Pauthe and Roussel, 1952; Campbell, Hancock and Rothschild, 1953); microscopic assessment of the degree of motility, either directly in semen (Harvey and Jackson, 1945; McLeod, 1946*a*; Emmens, 1947; Farris, 1950) or by the 'cervical mucus penetration test', in which a drop of semen is placed on the microscope slide next to cervical mucus and the passage of spermatozoa through the mucus is followed by microscopic observation (Barton and Wiesner, 1946; Harvey and Jackson, 1948). The determination of the concentration of motile spermatozoa in a semen sample is generally held to be the criterion most clearly correlated with the actual fertility rate; but even this relationship occasionally fails to give a true picture, and motile spermatozoa are by no means always fertile.

Physico-chemical methods of semen analysis depend on the determination of a wide range of physical, chemical and metabolic characteristics of semen, related to the physiological function of the sperm and the seminal plasma. Here belong methods for the measurement of certain optical properties of semen such as the light-scattering and light-absorption power, electrical conductivity and impedance changes; specific gravity, osmotic pressure; hydrogen ion concentration, buffering capacity; occurrence of semen-specific metabolites such as fructose, citric acid, ergothioneine, and inositol, and various enzymes such as hyaluronidase and certain phosphatases; and finally, the rate of anaerobic and aerobic metabolic processes in semen expressed in terms of fructolysis index, respiration rate or methylene-blue reduction time.

Optical and electrical properties of semen

An optical property closely related to sperm concentration is the turbidity of semen. In the past, determinations of sperm concentration were based on microscopic sperm-counts but more recently these have been partly replaced by turbidimetric measurements, which can be carried out quickly with suitably diluted samples either in a visual comparator by direct comparison with opacity standards, or in a photoelectric absorptiometer, to provide values for the relative light transmission of semen (Burbank, 1935; Comstock and Green, 1939; Henle and Zittle, 1942; Salisbury *et al.* 1943; Rothschild, 1950*b*). The turbidimetric methods, however, it must be

remembered, rest on the assumption that although light is absorbed both by sperm and seminal plasma, the scattering of light is due exclusively to the former, so that only light-scattering as such, is related directly to sperm density. In practice, the light-scattering and light-absorption due to the spermatozoa predominates so much over the light-absorption of the seminal plasma that under properly controlled conditions the error due to the presence of the latter can be neglected. This applies certainly to the semen of sea-urchin, bull and ram, but need not necessarily be true of other species, in which the seminal plasma itself shows a considerable degree of opaqueness. Furthermore, the spermatozoa present in a given ejaculate may differ in their light-reflecting capacity, apparently in proportion to the degree of sperm 'ripeness' and thereby, of fertility (Lindahl *et al.*, 1952). The light-reflecting capacity of the sperm cell may also be related to another optical change associated with sperm ripening, namely the increase in 'luminosity' of the sperm surface in dark-field illumination.

It has been claimed that the spermatozoon possesses at the head and tail small, but directly opposite, electrical charges. However, all that can be claimed with certainty is that an electric charge is associated with the sperm cell but that its sign and magnitude depend largely on the concentration of the various positively and negatively charged ions in the surrounding medium. The following values for electro-conductivity in semen at 25°, expressed in reciprocal ohms$\times 10^{-4}$, were given by Bernstein and Shergin (1936): bull 89·5–116·3, ram 48·5–80·5, stallion 111·3–129·5, boar 123·3–134·6, rabbit 85·5–101·4; and by Zagami (1939): man 88–107 (at 20°), and dog 129–138.

Much scientific interest and practical importance in the rating of semen quality attaches to the characteristic periodic changes in electrical impedance which occur in semen samples with high sperm density and motility, and which have been shown by Rothschild (1948*b*, 1949, 1950*a*) to be associated closely with the so-called 'wave motion' of the spermatozoa. When a drop of ram or bull semen of high density is placed on a microscope slide and examined at 37° under low magnification, a characteristic phenomenon can be observed in the form of slow, periodically appearing bands of high opacity or 'waves'. Measurements by means of the impedance

bridge make it possible to assess this characteristic sperm movement in a more objective and quantitative manner than by visual estimates; but they can only be made with semen samples which are sufficiently dense to show the 'wave motion'. Assays carried out with the electrical method in the semen of bulls at several British Artificial Insemination Centres revealed an interesting correlation of impedance change frequency with the conception rate (Bishop *et al.*, 1954).

Distinct from the wave-motion or locomotion 'en masse' (Walton, 1952) is the movement of individual spermatozoa which in highly motile semen takes the form of so-called 'forward' or 'progressive' motility, but which in poor specimens is confined to side-to-side 'oscillatory' movements (Plate IV). Recently, there have been several attempts to replace the subjective and semi-quantitative microscope appraisals of motility by more clearly defined methods, of which those by Bosselaar and Spronk (1952) and Rothschild (1953*a*, *b*) deserve special mention.

Viscosity, specific gravity, osmotic pressure, and ionic equilibrium

The viscosity of whole semen depends largely upon the concentration of spermatozoa. Thus for example, the viscosity of bull semen (relative to that of pure water which is taken as unity) can vary from 1·76 in a specimen containing 80,000 sperm/μl. to 10·52 in a sample with 2,260,000 sperm/μl. (Szumowski, 1948). Seminal plasma itself, seldom exceeds in bulls a relative viscosity value of 2 but higher values have been recorded in other species, especially those which exhibit the phenomenon of gelation.

The average specific gravity of whole semen is 1·028 in man, 1·011 in dog, and 1·035 in bull, with fluctuations due in the first place to the variable ratio between sperm and seminal plasma. The latter is so much lighter than the spermatozoa that in practice the specific gravity of semen is often found to be directly proportional to sperm concentration. In bull semen, low specific gravity is usually associated with low sperm concentration and poor 'quality', whereas high values accompany good density and good 'quality' (Anderson, 1946*a*). This is not surprising in view of the fact that the specific gravity of bull seminal plasma is not greater than that of blood plasma, whereas the average specific gravity of bull spermatozoa

PLATE IV



TRACK OF THE SPERM-HEAD

Cinematograph of a bull spermatozoon (in semen diluted 1 : 450) moving forward at a speed of about 0·15 mm./sec. Photographic plate exposed for 1 sec., using dark ground illumination. Mag.×673. Only the projection of the movement of the sperm-head is seen, the tail leaving no track. As the sperm-head is shaped like an elliptical disc, intense light scattering occurs only when the thin edge of the head is visible; during 1 sec. exposure ten images of the head were recorded which means that it rotated or oscillated backwards and forwards ten times.

(By courtesy of Lord Rothschild)

(1·28) considerably exceeds that of the erythrocytes (1·1) and other cells of the animal body. As already mentioned (p. 8), Lindahl and Kihlström (1952) believe that the wide range of variations in the specific gravity of ejaculated bull spermatozoa (1·240–1·334) is due to the variable proportion of 'ripe' (heavier) and 'unripe' (lighter) sperm cells in semen. However, whereas the specific gravity of the sperm cell is due to the highly condensed nuclear and protoplasmic protein constituents, the specific gravity of the seminal plasma is the direct outcome of the actual osmotic pressure exerted by electrolytes and is thus related to the depression of the freezing point.

Determinations of the osmotic pressure in terms of freezing point depression have been carried out in the semen of several species and the following data (in centigrade) are available: man 0·55–0·58, bull 0·54–0·73, ram 0·55–0·70, stallion 0·58–0·62, jackass 0·56–0·62, boar 0·59–0·63, dog 0·58–0·60, rabbit 0·55–0·59 (Slovtzov, 1916; Roemmele, 1927; Milovanov, 1934; Bernstein and Shergin, 1936; Zagami, 1940; Salisbury, Knodt and Bratton, 1948; Nishikawa and Waide, 1951). It would seem that generally, more reliance should be placed on results obtained with seminal plasma than with whole semen. More recently, Rothschild and Barnes (1954) carried out freezing point determinations on forty samples of seminal plasma from ten bulls of different breeds, and obtained a mean value of 0·53 with a standard error of 0·005.

The electrolytes in the seminal plasma are those made available by the secretions of the male accessory organs. Additional information on the content of the inorganic constituents in semen is summarized in Table 4, but again, owing to the variable composition of semen, the chemical data relating to whole semen must be taken with due reservations.

The interrelations between the various ions in semen differ from those existing in blood in several respects, but most perhaps because of the presence of a much higher concentration of extracellular potassium and a correspondingly lower content of sodium. Miescher (1897) who was the first to examine systematically the chemical composition of salmon semen, found in the ash prepared from the seminal plasma (parts/100): 51·0 NaCl, 8·2 KCl, 14·0 K_2SO_4, and 26·8 Na_2CO_3. In the trout, Schlenk (1933) recorded a value of 80 mg. K/100 ml. semen. In the sea-urchin (*Echinus esculentus*) Rothschild

(1948*c*) found 155 mg. K/100 ml. seminal plasma. In the higher mammals, the content of potassium in semen may reach 400 mg./100 ml. (Table 4); it is derived mainly from the seminal plasma where it is found at least partly, in association with citric acid (see p. 188).

Hydrogen ion concentration and buffering capacity

The reaction of freshly ejaculated semen is not far from neutral (Table 8). On standing, the semen may become alkaline at first, unless precautions are taken to prevent the loss of carbon dioxide,

TABLE 8. *Hydrogen ion concentration in semen*

Species	pH	References
Boar	7·3 –7·9	McKenzie, Miller and Bauguess (1938)
Bull	6·4 –7·8	Anderson (1942); Hatziolos (1937)
	6·48–6·99	Laing (1945)
Cock	7·02–7·18	Zagami (1939)
	6·3 –7·8	Lambert and McKenzie (1940)
Dog	6·67–6·76	Zagami (1939)
Fox	6·2 –6·4	Starkov (1934)
Man	7·35–7·50	Zagami (1939)
	7·05–7·41	Huggins, Scott and Heinen (1942)
Rabbit	6·59–6·86	Zagami (1939)
	6·8 –7·5	Lambert and McKenzie (1940)
Ram	5·9 –7·3	McKenzie and Berliner (1937)
Stallion	6·2 –7·8	Nishikawa and Waide (1951)
Arbacia punctulata	7·6 –7·9	Hayashi (1945)
Echinus esculentus	7·5	Rothschild (1948*c*)

but later this change is followed, at least in those specimens which contain fructose and a high concentration of spermatozoa, by a rapid decrease of pH, owing to fructolysis and accumulation of lactic acid. Excessive initial alkalinity of semen in some species, notably in bulls and rams, often accompanies low fertility, the alkaline reaction being associated with absence or low concentration of sperm and with a correspondingly higher proportion of seminal plasma. A significant negative correlation between the pH value and sperm density and motility has been noted frequently in bulls and rams (cf. Anderson, 1945); in the latter, according to McKenzie and Berliner (1937), normal semen is slightly acid or at

any rate never more alkaline than pH 7·3, whereas in sterile rams the pH value may reach 8·6. However, the rate at which acidity increases after ejaculation is much more significant for the assessment of semen than the initial pH value, because it is related directly to the actual glycolytic activity of the spermatozoa, and indirectly to sperm density and motility. In bull and ram semen this correlation is said to be so close that the decrease in pH on incubation can serve as an additional indicator of semen quality (cf. Anderson, 1945; Laing, 1945; Reid, Ward and Salsbury, 1948*b*). As a matter of fact, however, the significance of this method is limited because as the pH value falls, the semen becomes too acid for spermatozoa to maintain their motility and metabolism.

Some of the effects which are due to variations in hydrogen ion concentration in semen will be discussed in the next chapter; here we shall concern ourselves with the buffering capacity of semen which has been the subject of some interesting work, especially in bull and human semen (Shergin, 1935; Smith and Asdell, 1940; Easley, Mayer and Bogart, 1942; Sheldovsky, Belcher and Levenstein, 1942; Willett and Salisbury, 1942; Anderson, 1946*a*). On the whole, bull semen is more highly buffered on the acid than on the alkaline side, and its normal buffering capacity depends mainly upon citrate and bicarbonate, but not phosphate. Anderson used 107 specimens of seminal plasma from normal bulls and measured their buffering power by adding 0·05 ml. 0·1N-HCl to 0·15 ml. plasma, and determining the pH with the help of a glass electrode, before and after acid addition; the decrease in pH value obtained in this way was $1{\cdot}84 \pm 0{\cdot}038$. He also investigated the relationship between buffering capacity and the period for which sperm motility of 70% and over, was maintained; semen specimens which kept up this motility for 24 hr. and upward, all had a good and fairly uniform buffering capacity (decrease in pH=1·74), but those with a smaller degree of motility at 24 hr. had a poorer buffering capacity (decrease in pH=2·00).

Metabolism of semen, and its relation to sperm density and motility

The two chief metabolic processes of semen, namely fructolysis and respiration, are both a direct outcome of the metabolic activity of the sperm cells; their rate is determined largely by the number

of spermatozoa in the semen and the degree of sperm motility (Mann, 1949). The more chemical aspects of semen metabolism will be discussed fully in conjunction with specific groups of substances metabolized by spermatozoa, such as sugars, lipids and amino acids. Here, only the general outline of sperm metabolism will be given, in so far as it helps to bring out the relationship between the metabolic processes and other characteristics of semen, in particular, sperm concentration and motility.

Glycolysis

In the absence of oxygen, for example under the conditions of semen storage for artificial insemination, the spermatozoa rely on carbohydrate metabolism as the chief source of energy. Even before the identity of the seminal sugar was revealed, the rate of lactic acid production or 'glycolysis' was used as a method for semen appraisal (Comstock, 1939; Webster, 1939; MacLeod, 1941*a*, *b*, 1943*b*; Moore and Mayer, 1941; Comstock, Green, Winters and Nordskog, 1943; Laing, 1945; Salisbury, 1946; Westgren, 1946.) However, the discovery of fructose and the work on fructolysis made available a chemical approach to several practical problems of male fertility (Mann, 1946*b*, 1948*a*, *b*, 1949; Mann, Davies and Humphrey, 1949; Mann and Lutwak-Mann, 1951*a*, *b*; Mann and Parsons, 1950; Mann and Walton, 1953).

The anaerobic incubation of freshly ejaculated semen is accompanied by a progressive decline in the content of fructose with a simultaneous accumulation of lactic acid; in the presence of suitable buffer, the process of fructolysis in semen with good sperm motility can be shown to progress almost linearly until practically all of the sugar is used up (Fig. 8). On this basis a photometric method has been worked out for the measurement of sperm fructolysis, and the 'index of fructolysis' has been defined as the amount of fructose (in mg.) utilized by 10^9 spermatozoa in 1 hr. at 37° (Mann, 1948*a*, *b*). In normal bull semen, the index of fructolysis is about 1·4–2 but it varies, and is significantly correlated with both the concentration and the motility of spermatozoa. Fructose is not utilized by either azoospermic semen, i.e. ejaculates devoid of sperm, or by necrospermic semen, containing immotile spermatozoa. The existence of a positive correlation between the rate of fructolysis and the

concentration of motile spermatozoa has been amply confirmed by studies on both bull and human semen (Anderson, 1946*b*; Eichenberger and Goossens, 1950; Bishop *et al.*, 1954) but the existence of a similar correlation between the rate of fructolysis and fertility

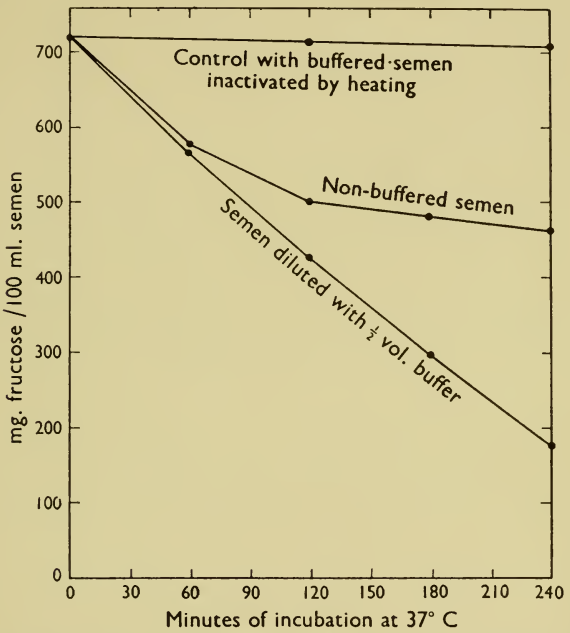


FIG. 8. *Fructolysis in bull semen* (920,000 *sperm/μl.*) *incubated at* 37°; the disappearance of fructose was measured in (i) bull semen which has been diluted with half a volume 0·25M phosphate buffer pH 7·4, (ii) unbuffered semen, and (iii) buffered semen inactivated by heating.

(Mann, 1948a)

is still a matter of dispute; it has been claimed for bull semen by some authors (Gassner, Hill and Sulzberger, 1952) but was denied by others (Bishop *et al.*, 1954). In both cases, however, the material used for the survey consisted mainly of bulls kept for breeding purposes at Artificial Insemination Centres. More insight into the relationship between fructolysis and fertility could probably be gained by studies on semen from bulls of subnormal fertility.

A lowered rate of fructolysis has been observed in certain cases of human subfertility (Davis and McCune, 1950; Birnberg, Sherber and Kurzrok, 1952). However, in human semen with its physiologically low sperm density but high fructose content, it is actually more convenient to assess the rate of fructose utilization by measuring the formation of lactic acid (chemically or manometrically) rather than the disappearance of sugar. In any case, if one were to measure fructolysis in human semen by the disappearance of sugar, it would be essential to use a photometric method specific for fructose, and not base the results merely upon the changes in 'reducing value', because human semen contains a fair amount of reducing substances other than carbohydrate, which represent a substantial proportion of the total 'reducing value' towards reagents such as cupric hydroxide, ferricyanide etc. Moreover, the content of these reducing substances often increases during the incubation of semen, thus rendering unreliable, not to say senseless, determinations of fructose based upon reduction measurements.

Methylene-blue reduction test

A method often used in the evaluation of semen quality is the 'methylene-blue reduction test' which is the outcome of dehydrogenase activity of the semen and depends on the determination of the time which it takes a semen sample to decolorize a certain amount of methylene blue, under standard conditions of incubation *in vitro* (Sørensen, 1942; Beck and Salisbury, 1943; VanDemark, Mercier and Salisbury, 1945; Boenner, 1947). In Sørensen's original method the incubation was carried out in a Thunberg tube, but in Russia (Milovanov and Sokolovskaya, 1947) and in France (Brochart, 1948*b*), the test was later performed by introducing a drop of semen mixed with methylene blue into a capillary tube and by observing the decolorization in the central portion of the column; in good-quality bull semen, with high density and motility, decolorization usually takes place within less than 10 min. at 20°; if it extends beyond 30 min., it signifies poor semen quality. However, though useful, the test is of limited scope, not least because as long ago as 1941, Lardy and Phillips showed that the reduction of methylene blue by sperm suspensions may be markedly delayed by a variety of substances including glucose, lactate and citrate.

Respiration

In the presence of oxygen, semen shows a considerable respiratory activity which is correlated both with concentration and motility of spermatozoa. It is usual to express sperm respiration in terms of Z_{O_2}, a coefficient introduced by Redenz (1933) to denote μl. O_2 taken up by 10^8 sperm cells during 1 hr. at 37°; Z_{O_2} values reported by Lardy and Phillips (1943*a*) for bull, cock, rabbit and ram semen, are 21, 7, 11 and 22, respectively. The use of Z_{O_2} is more convenient than Q_{O_2} since the latter involves centrifugation of semen and washing of sperm with an unphysiological fluid such as distilled water; the average Q_{O_2} of ram sperm based on the dry weight of washed sperm is about 8. Further references to the subject of sperm respiration measurements, classified according to species are listed in Table 9.

Chang and Walton (1940) found a close relationship between motility and respiratory activity in ram sperm. Walton (1938) suggested measurements of oxygen uptake in bull semen as a supporting method for the assessment of semen quality. Walton and Edwards (1938) compared the breeding records of thirteen bulls, taking as a measure of their fertility the number of matings required to produce pregnancy in cows; when they analysed ten different samples of semen from each of these bulls, they found that there is a close correlation between the respiratory activity of semen and fertility assessed on the natural service records. But Ghosh, Casida and Lardy (1949), and Bishop *et al.*, (1954) failed to establish a correlation between the respiratory activity of bull semen and fertility, as assessed on the basis of artificial insemination records.

Further study will probably clear up these uncertainties but in general, when we consider the significance of sperm respiration measurements, there are certain points which must be taken into account. Unlike fructolysis, the respiratory activity of semen is not entirely exogenous since it involves, in addition to the oxidative removal of products of fructolysis (chiefly lactic acid), the endogenous respiration, i.e. oxidation of some intracellular reserve material, most probably a lipid. Moreover, it is possible to create, experimentally at any rate, conditions under which sperm respiration can be dissociated from motility. For example, in the presence

TABLE 9. *List of some references to work on sperm respiration*

BOAR
Winchester and McKenzie, 1941.

BULL
Redenz, 1933; Windstosser, 1935; Henle and Zittle, 1941, 1942; Lardy and Phillips, 1943*a*, *b*, 1944; Lardy, Hansen and Phillips, 1945; Tosic and Walton, 1950; Schultze and Mahler, 1952; Bishop and Salisbury, 1954; Melrose and Terner, 1953; Bishop, Campbell, Hancock and Walton, 1954.

COCK
Winberg, 1939; Lardy and Phillips, 1943*a*; Kosin, 1944.

DOG
Ivanov, 1931; Bishop, 1942.

FOX
Bishop, 1942.

MAN
McLeod, 1939, 1943*a*, *b*; Shettles, 1940.

RABBIT
Lardy and Phillips, 1943*a*; White, 1953.

RAM
Ivanov, 1936; Comstock, 1939; Chang and Walton, 1940; Comstock, Green, Winters and Nordskog, 1943; Lardy, Winchester and Phillips, 1945; Mann, 1945*a*; Mann and Lutwak-Mann, 1948; White, 1953.

OYSTER, *Saxostrea commercialis*
Humphrey, 1950.

SEA-URCHINS
Warburg, 1915; Gray, 1928, 1931; Barron *et al.*, 1941, 1948, 1949; Hayashi, 1946; Rothschild, 1948*a*, *c*, *d*, 1950*d*, 1951*b*; Spikes, 1949.

of a suitable concentration of fluoride, one can abolish both motility and fructolysis in ram spermatozoa without greatly suppressing the respiration (Mann and Lutwak-Mann, 1948) (Fig. 9). Another example is provided by the response of ram sperm to succinate. Thus, whereas the oxygen consumption of *intact* ram spermatozoa is not enhanced markedly by the addition of succinate, sperm cells treated with spermicidal detergents such as cetyltrimethylammonium bromide, 2-phenoxyethanol, sodium dodecylsulphate and similar surface-active agents, show in the presence of succinate a high rate of oxygen uptake although of course, the motility and the fructolysis have been completely abolished (Koefoed-Johnsen and Mann, 1954).

The effect of fluoride on fructolysis is due chiefly to the inhibition of enolase; the addition of pyruvate to fluoride-treated spermatozoa

enables the lactic acid formation to continue (Ivanov, 1943; Lardy and Phillips, 1943*c*; Mann, 1945*b*; Melrose and Terner, 1952, 1953). But according to the last-named authors, washed bull spermatozoa

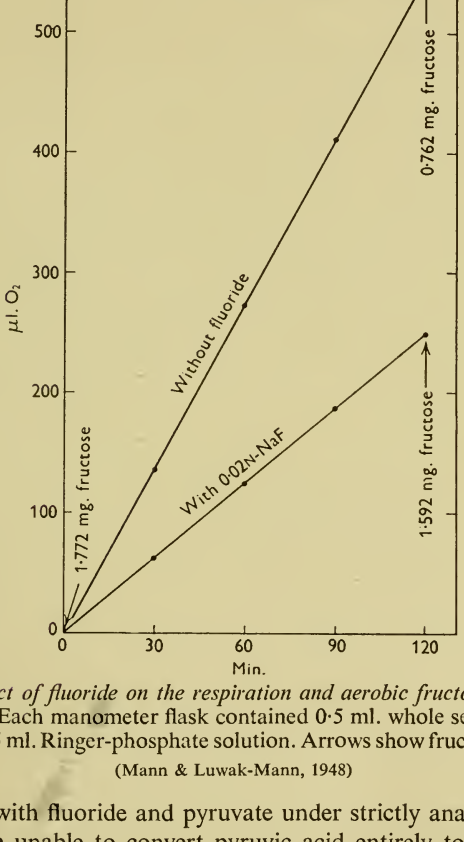


FIG. 9. *Effect of fluoride on the respiration and aerobic fructolysis of ram semen.* Each manometer flask contained 0·5 ml. whole semen diluted with 1·5 ml. Ringer-phosphate solution. Arrows show fructose content.

(Mann & Luwak-Mann, 1948)

incubated with fluoride and pyruvate under strictly anaerobic conditions, are unable to convert pyruvic acid entirely to lactic acid but instead, a dismutation takes place in which of two molecules pyruvic acid, only one is reduced to lactic acid, and the other is oxidized to carbon dioxide and acetic acid:

$$2CH_3{\cdot}CO{\cdot}COOH + H_2O \longrightarrow CH_3{\cdot}CHOH{\cdot}COOH + CO_2 + CH_3COOH$$

Aerobically, in the presence of both fluoride (0·02M) and pyruvate (0·01M), bull spermatozoa consume less oxygen than without fluoride; this low rate of oxygen uptake can be enhanced by the addition of 2 : 4-dinitrophenol (10^{-4}M), which brings about a more complete oxidation of that fraction of pyruvate which is not reduced to lactic acid. Melrose and Terner (1952, 1953) claim that bull semen can be graded according to the respiratory response of washed spermatozoa to a system made up of fluoride, pyruvate and dinitrophenol, and that in highly fertile samples the oxygen consumption is low in the presence of pyruvate and fluoride, but is increased two-fold or more, by the addition of dinitrophenol.

Among substances which can provide exogenous material for sperm respiration are glycolysable sugars and lower fatty acids such as lactic, pyruvic and acetic acid (Fig. 10) but the species differences in this respect are very marked. Thus, while in mammalian spermatozoa for example, it is possible to prolong the respiratory activity for a considerable length of time with glucose, fructose, mannose, L(+)-lactate, pyruvate, propionate, butyrate, and oxaloacetate (Lardy and Phillips, 1944, 1945; Mann and Lutwak-Mann, 1948; Humphrey and Mann, 1949; Tosic and Walton, 1950; Melrose and Terner, 1953), the respiration of oyster spermatozoa is increased by α-oxoglutarate and oxaloacetate, decreased by acetate, propionate and butyrate, and remains unaffected by lactate, glucose, fructose and mannose (Humphrey, 1950). It appears also that the mode of action of several organic acids on the respiration of mammalian spermatozoa differs fundamentally from the influence exerted by the same substances on the sperm of lower animals. This is illustrated best by the example of the peculiar response of sea-urchin sperm to malonate (0·03M), which was reported by Barron and Goldinger (1941*b*) to increase both the O_2 uptake and the aerobic CO_2 output of sperm by as much as 200%. Succinic acid is another example of a substance which was found to be highly effective in the respiration of sea-urchin sperm (Barron and Goldinger, 1941*a*; Goldinger and Barron, 1946) but has little effect on the oxygen uptake of intact mammalian spermatozoa. Another characteristic difference in behaviour between sea-urchin and mammalian sperm concerns the effect of the fatty acids on the *initial* rate of sperm respiration. In the case of sea-urchin spermatozoa, the addition of a fatty acid salt

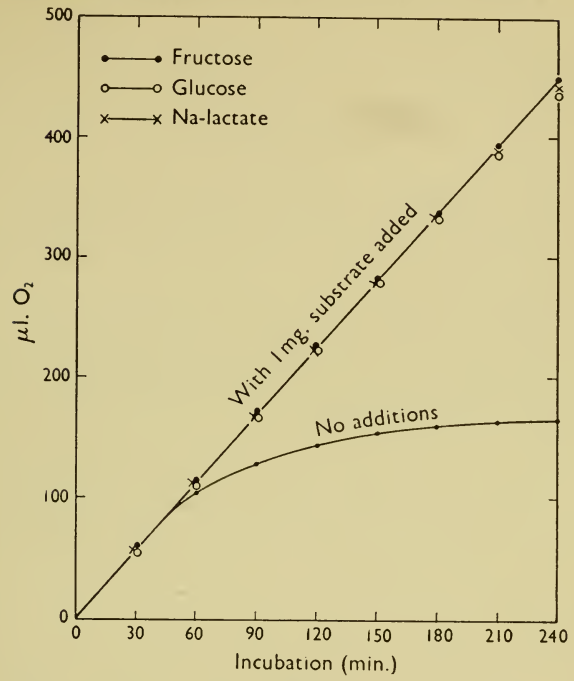


FIG. 10. *Effect of fructose, glucose and lactate on the respiration of washed ram spermatozoa* (the same effect was obtained also with acetate and pyruvate); oxygen uptake of 3 ml. buffered suspension of $0{\cdot}45 \times 10^9$ spermatozoa.

(Mann & Lutwak-Mann, 1948)

often produces a prompt and marked rise in the initial rate of O_2 uptake. In our experience, however, the addition of organic acid salts to washed suspensions of mammalian spermatozoa, does not lead necessarily to an actual increase in the initial O_2 uptake but instead, these substances act by maintaining and prolonging the initial rate of respiration, thereby delaying the decline in O_2 consumption which would set in otherwise (Mann, 1949). Furthermore, by adding the same substances to respiring suspensions of washed mammalian sperm at a stage when the respiration had already begun to decline, one can prevent a further deterioration in the rate of O_2 consumption.

CHAPTER III

The Influence of Extraneous Factors, Hormones, and Environmental Conditions

Sperm inhibitors and spermicidal substances. Chemical aspects of short-wave radiation. Variations in hydrogen ion concentration and tonicity. Influence of heat and cold; sperm vitrification and 'la vie latente'. Role of hormones. Sperm-egg interacting substances and chemotaxis. 'Dilution effect' and chemical changes associated with senescence. The use of artificial diluents in the storage of semen.

The list of agents, both physical and chemical, which affect spermatozoa, includes among others, changes in temperature, visible light, short-wave radiation, atmospheric pressure, ionic strength, and a host of pharmacologically active substances. The vast literature on the subject of sperm activation and inhibition goes back as far as Leeuwenhoek's observation that dilution with rain water deprives the canine 'animalculi' of motion, and a report by his learned friend Johan Ham of Arnhem, on the loss of sperm motility in a patient dosed with turpentine. Among Spallanzani's contributions in this field is the discovery that freezing in snow does not necessarily kill the 'spermatic vermiculi' but reduces them to a state of 'lethargy' from which they recover when returned to higher temperature. The XIXth century abounds in studies on the effect of changes in the medium on sperm motility and survival. Prévost and Dumas (1824) extended Spallanzani's observations on the lethal effect of electric shock and certain poisons; Donné (1837) investigated the influence of milk, urine, and the vaginal and cervical secretions; de Quatrefages (1850, 1853) described in great detail the marked toxicity of copper, lead and mercuric salts. Newport (1853) studied the narcotizing influence of chloroform vapours on the amphibian spermatozoa, and concluded 'that the spermatozoon does not impregnate when entirely deprived of its power of motion by narcotization and disenabled to penetrate into the envelopes of the egg'. Both Newport

and de Quatrefages were fully aware of the fact that sodium chloride and various other sodium and potassium salts, are able to stimulate or inhibit sperm motility, according to concentration and specific experimental conditions. Their results were soon confirmed and extended by others, including Koelliker (1856) whose paper on 'Physiologische Studien über die Samenflüssigkeit' still remains the most comprehensive survey of its kind, and includes observations on spermatozoa of the bull, stallion, dog, rabbit, pigeon, frog and fish. Koelliker pointed out that spermatozoa rendered motionless by dilution with water can be revived by prompt addition of salts or concentrated solutions of certain organic substances such as sucrose, glucose, lactose, glycerol, urea and various proteins. He investigated in some detail the activating influence of blood serum, male accessory gland secretions, and of a variety of inorganic and organic substances on sperm motility. It was he who found that cyanide is not an inhibitor of sperm motility and established that acids are, on the whole, more harmful to the sperm than alkalies.

Furthermore, Koelliker noticed that if a drop of a fairly concentrated solution of potassium hydroxide is mixed with a drop of semen on a microscopic slide, there is usually a sudden outburst of activity before the spermatozoa are rendered motionless. Such a period of short-lived stimulation which precedes the terminal loss of activity, is rather characteristic of various sperm-paralysing agents including distilled water. Schlenk (1933) aptly named the phenomenon 'Todeszuckung'. Not all investigators, however, seem to have realized the fundamental difference between short and prolonged activation phenomena, and many of them tended to confuse a transient increase in initial motility with the state of activity essential for the maintenance of continuous motility and for sperm survival. Only too often substances have been pronounced as beneficial to spermatozoa merely because they were observed to stimulate motility and metabolism, no heed being paid to the fact that this very stimulation may have shortened, rather than prolonged, the life of spermatozoa. Similarly, many a substance has been declared detrimental to spermatozoa solely because it appeared to reduce the speed of movement and metabolic rate. However, quite often the lowering of activity tends to prolong the life of spermatozoa, and favours, rather than hinders, their survival.

Sperm inhibitors and spermicidal substances

So far, detailed studies on the mechanism which underlies the action of sperm inhibitors, have been relatively limited in scope and concerned largely with chemical compounds which affect respiration and glycolysis, among them cyanide, azide, dinitrophenol, and fluoride, or which combine with sulphydryl groups, e.g. iodoacetate, iodoacetamide, *o*-iodosobenzoate, and *p*-chloromercuribenzoate. Even these studies, however, have clearly indicated the existence of remarkable species differences in sperm behaviour. Thus for instance, iodoacetate which is one of the strongest inhibitors of sperm activity in higher animals, has a pronounced stimulating action on the oxygen uptake of sea-urchin spermatozoa; this peculiar effect of iodoacetate is shared by other sulphydryl-binding compounds, as well as by malonate and nitrogen mustard (Barron and Goldinger, 1941*b*; Barron, Nelson and Ardao, 1948; Barron, Seegmiller, Mendes and Narahara, 1948). It is interesting and important to note that widely divergent results may be attained with a given substance according to a particular set of experimental conditions: rabbit spermatozoa, washed and resuspended in a sugar-free isotonic medium, are immobilized completely by 0·0001M 2 : 3 : 5-triphenyltetrazolium chloride, but a 200 times higher concentration of this substance is ineffective towards sperm suspended in a glucose-containing medium (Bishop and Mathews, 1952). It is equally salutary to bear in mind that a substance which does not increase the initial rate of sperm activity, may nevertheless be utilized by the spermatozoa as an essential nutrient. For instance, most of the sugars and fatty acids which are oxidized by spermatozoa, do not act by increasing the initial rate of respiration but by maintaining it.

Surprisingly enough, many substances endowed with pronounced pharmocological action in the whole animal, such as the alkaloids, appear to exert little or no effect upon spermatozoa *in vitro*. Sperm cells are also remarkably resistant to ethanol. Ivanov (1913) observed excellent motility in dog sperm to which he added 2–5% ethanol, and he managed to obtain live and normal offspring from an animal inseminated with semen mixed with 10% ethanol. It may be mentioned here that ethanol is one of the substances which are definitely known to pass into semen after ingestion by the animal (Farrell,

1938); sulphonamides provide a similar example (Farrell, Lyman and Youmans, 1938; Kuehnau, 1939; Hug, 1940).

Marked spermicidal power is characteristic of a great many surface-active agents, quinones and heavy metal compounds. A technique for assessing the spermicidal activity of pure substances has been developed by Baker (1931, 1932, 1935) whose so-called 'killing concentration' is the lowest one capable of killing all spermatozoa suspended in a buffered glucose-saline solution (Baker's solution) within half an hour, at body temperature, under standard conditions *in vitro*. Of the many substances examined by Baker, the most highly spermicidal was phenylmercuric acetate (killing concentration 0·001%). Various quinones such as toluquinone, butylquinone, methoxyquinone, parabenzoquinone, ethylquinone and paraxyloquinone were also strongly spermicidal. Lower down the scale were mercuric chloride, methoxyhydroquinone, formaldehyde, methylhydroquinone, saponin, and hexylresorcinol. A critical account of the existing methods for testing the efficiency of spermicidal compounds is given by Millman (1952), Davidson (1953) and Gamble (1953).

As to the mode of action of surface-active agents, such as e.g. cetyltrimethylammonium bromide, cetyldimethylbenzylammonium chloride, *p*-tri*iso*propylphenoxypolyethoxyethanol, dodecyl sulphate, various condensation products of long-chain fatty alcohols with ethylene oxide, and the numerous other ionic and non-ionic detergents, there is some evidence to show that these substances act directly on the constituents of the so-called lipid capsule, i.e. the lipid-containing outer layer which protects the surface of the spermatozoon. The mechanism of the spermicidal activity of detergents on the sperm may be likened to the haemolytic action of surface-active compounds on the erythrocytes, or the bactericidal effects of these substances on various microorganisms. The changes brought about by detergents manifest themselves in a loss of motility and fructolytic power, and in a grossly altered permeability of the sperm cells as indicated by the leakage of cytochrome *c*. In contrast to intact spermatozoa, the respiration of sperm cells treated with suitable concentrations of detergents is markedly increased by succinate (Koefoed-Johnsen and Mann, 1954).

As to the action of some at any rate, of the many other

spermicidal substances, including organo-metallic compounds, there are indications that it is due to the blockage of vital sulphydryl groups in the spermatozoa. Thus for example, the immobilizing effect of iodoacetate on bovine spermatozoa, which has been studied by Lardy and Phillips (1943*b*), is most probably due to the sulphydryl group-binding capacity of this substance. In a war-time study MacLeod (1946*b*) showed that the inhibition of the metabolism and motility of human spermatozoa by organic arsenicals can be overcome by 1 : 2 : 3-trithiolpropane, and that the inhibitory effect of cupric ions can be prevented by the addition of cysteine or glutathione (MacLeod, 1951). Researches by Mann and Leone (1953) have shown that both motility and fructolysis are abolished in mammalian spermatozoa by several thiol reagents, including cupric ions, hydrogen peroxide and *o*-iodobenzoate but that ergothioneine, a normal constituent of boar seminal plasma, can efficiently counteract the paralysing action of these reagents. The oxidation of sulphydryl groups probably explains also the spermicidal action of hydrogen peroxide. The toxicity of hydrogen peroxide to spermatozoa, noted already by Guenther (1907), is of particular interest since this substance can actually be formed under certain conditions by the spermatozoa themselves, in the course of their aerobic metabolism, and is responsible for the so-called oxygen damage which occurs as a result of oxygenation of semen (MacLeod, 1943*a*; Tosic and Walton, 1946*a*, *b*; VanDemark, Salisbury and Bratton, 1949). Yet another aspect of the damaging action of hydrogen peroxide has been revealed by studies on the effect of X-rays on spermatozoa. When sea-water heavily irradiated with X-rays was used as a diluent for sea-urchin semen, there was a great reduction in the survival period of spermatozoa and a considerable delay in the cleavage of the eggs fertilized with these spermatozoa. The responsible toxic agent present in the irradiated sea-water has been identified by Evans (1947) as hydrogen peroxide. It is only fair to add, however, that not all investigators agree with the conclusion that the toxicity of irradiated media is due exclusively to hydrogen peroxide.

Chemical aspects of short-wave radiation

The chemical changes underlying the action of X-rays on spermatozoa still remain largely unexplored, and the precise targets of this

and other forms of radiation in the sperm cell are by no means established. The amount of information on this subject, however, is steadily mounting, ever since Bohn's (1903) and Hertwig's (1911) fundamental observations on the abnormal development of sea-urchin and frog ova inseminated with spermatozoa previously exposed to radium emanation. The exposure of the testes to relatively small doses of X-ray results in sterility because of the extreme sensitivity of the seminiferous epithelium and complete breakdown of spermatogenesis; but direct irradiation of ripe, ejaculated spermatozoa, in which the nuclei are in the resting state, has usually little or no effect on motility, longevity, morphology or metabolism of sperm. Nevertheless, irradiated spermatozoa are either altogether infertile or, if they retain the power to penetrate the ovum and effect syngamy, they are incapable of inducing normal development of the ovum owing to damaged chromatin. This conclusion is the outcome of extensive investigations on irradiated spermatozoa of several species, including sea-urchins (Henshaw, 1940; Barron, Gasvoda and Flood, 1949; Blum, 1951), frogs (Bardeen, 1907; Dalcq and Simon, 1931; Rugh, 1939), insects (Barth, 1929; Eker, 1937), rats (Henson, 1942; Fogg and Cowing, 1952), mice (Snell, 1935), rabbits (Asdell and Warren, 1931; Amoroso and Parkes, 1947; Murphree, Whitaker, Wilding and Rust, 1952), and fowl (Kosin, 1944). The irradiation of cock semen *in vitro* with X-ray doses up to 10,000 r has been shown by Kosin to have no detectable effect on the motility, respiration and anaerobic glycolysis, but the fertilizing capacity of these spermatozoa was markedly reduced already after exposure to 200 r, and was altogether destroyed after a dose of 5500 r. These results serve to underline the fact that sperm fertility may react to extraneous factors in a different manner from sperm motility and metabolism. In our experience, ram semen irradiated with 100,000 r and examined immediately after exposure, has normal motility, fructolysis and adenosine triphosphate content; evidently, the X-ray injury inflicted upon the spermatozoa as reflected in their diminished fertilizing capacity, must be the result of some other chemical change, possibly in the state of polymerization of the deoxyribonucleic acid in the sperm chromatin.

The sensitivity of the spermatogenic tissue to short-wave radiation is in marked contrast to the apparent resistance of the male accessory

glands which elaborate the seminal plasma. In rats after total body exposure to 500 r, there was occasionally a small but transient decrease in the level of fructose and citric acid secreted by the accessory organs but in surviving animals the activity was restored to normal within a few weeks after irradiation. This was a stage when the spermatozoa were mostly immotile and a large proportion of them severely damaged (Lutwak-Mann and Mann, 1950*b*).

Variations in hydrogen ion concentration and tonicity

Hydrogen ion concentration is undoubtedly one of the most important factors which influence the motility, viability and metabolism of spermatozoa in all species from sea-urchin to man (Cohn, 1917, 1918; Wolf, 1921; Gellhorn, 1920, 1927; Healy and Anderson, 1922; Mettenleiter, 1925; Barthélemy, 1926; Yamane and Kato, 1928; Komatsu, 1929; Schlenk, 1933; Grodziński and Marchlewski, 1935). Most authors agree that a value just above pH 7 provides the optimum for the survival of spermatozoa. Sperm respiration is stated to be optimal at the following pH values, boar 7·2–7·5, ram, 7·0–7·2, bull 6·9–7·0, cock 7·25, rabbit 6·8 (Winchester and McKenzie, 1941; Lardy and Phillips, 1943*a*). Below the optimum, motility and metabolism alike decline progressively. Alkalinity on the other hand, up to pH 8·5 and above, has frequently been observed to enhance the movement, particularly of human spermatozoa.

Whereas in some species including several fishes, the spermatozoa are known to be extremely sensitive to changes in pH, in others, e.g. in the frog, certain birds and mammals, they exhibit a remarkable degree of resistance (Gellhorn, 1920, 1922, 1927). In the case of rabbit sperm partially motile spermatozoa have been found within the range of pH 5·0–8·8 (Cole, Waletzky and Shackelford, 1940). More recently Emmens (1947) has shown that even at pH 9·5–10·0 rabbit spermatozoa retain partial motility for several hours, but they become immotile and die rapidly at pH values below 5·8. According to this author, the point at which the progressive movement is abolished and motility reduced to a condition where heads become completely stationary but tails still retain feeble motion, coincides with a state when about 50% of the sperm cells can be shown to be dead by the differential staining method of Lasley, Easley and McKenzie (1942); in this method, dead spermatozoa take

up eosin whereas live cells remain unstained. The time required to reach the 50% mortality level was stated to be 6 hr. at pH 6·4–6·5, 29 hr. at pH 7·2–7·9, 15 hr. at pH 8·5–9·5, 7 hr. at pH 9·7–9·8, and 4 hr. 30 min. at pH 10·2.

However, even after spermatozoa have been rendered immotile by excessive acidity they can still be resuscitated by alkalinization, always provided that the exposure to acid has not been unduly long (Engelmann, 1868; Lillie, 1913, 1919; Gray, 1915; Muschat, 1926; Schlenk, 1933). Under conditions *in vitro*, the time intervals at which the sperm can be revived, correlate well with the mortality rate (Emmens, 1947); no reactivation was seen when the death rate of rabbit sperm exceeded 80%.

The slowing down effect of weak acids and the reactivating influence of weak alkalis may well be of some importance for the activity of spermatozoa *in vivo*, in the various parts of the male and female reproductive tract. There are, however, several other important factors which influence sperm motility and survival *in vivo*, such as the concentration of various ions and nutrients, dilution, and the tension of oxygen. Thus, in the epididymis, and partly also in the vas deferens, the spermatozoa are immotile at a pH which approximates neutrality (Lanz, 1929; Bishop and Mathews, 1952); here, presumably, the combination of the very low oxygen tension, deficiency of carbohydrate and limitation of space acquires greater significance than the hydrogen ion concentration.

Tonicity as a sperm-affecting factor ranks equal in importance with the hydrogen ion concentration. Most investigators of semen, including Yamane (1920), Gellhorn (1922, 1924, 1927), and Milovanov (1934), agree that on the whole, spermatozoa seem to be immobilized much more readily by hypotonic than hypertonic, diluents. However, it must be remembered that the ultimate effect of tonicity depends upon certain other prevailing conditions. Thus, for example, Emmens (1948) who studied the motility of rabbit spermatozoa at various pH values with diluents of different chemical composition and tonicity, has shown that at pH 5·8–6·6, the spermatozoa were more sensitive to hypotonicity than to hypertonicity, but in an alkaline medium the situation was reversed. In an analogous study of ram, bull and human spermatozoa (Blackshaw and Emmens, 1951) it was established that at all pH levels, hypertonic solutions

were less harmful to motility than hypotonic media, and that the relatively slight adverse effect of hypertonicity could be diminished by partial replacement of sodium chloride with glucose. Furthermore, the extent to which a hypotonic or hypertonic medium can affect the spermatozoa, very much depends on the degree of sperm dilution. Highly concentrated solutions of sodium chloride incorporated in so-called salt-jellies, have been shown to possess marked spermicidal properties (Gamble, 1953).

Influence of heat and cold; sperm vitrification and 'la vie latente'

Temperature has long been known to exert a powerful influence in determining the onset of spermatogenesis and breeding activity in animals. The effect of increased temperature on the male reproductive organs presents many intriguing questions. Hyperpyrexia frequently causes a temporary azoospermia in man, and a hot climate is believed to be the principal cause of certain forms of subfertility among domestic animals in tropical countries. Mammalian testes removed from the scrotum and placed in the abdominal cavity, where the temperature is a few degrees higher, cease to produce spermatozoa; degeneration of the spermatogenic tissue sets in and spermatogenic function is not resumed while 'experimental cryptorchidism' prevails. In the guinea-pig, a complete cessation of spermatogenesis can be brought about experimentally by scrotal application of heat (6° above the normal body temperature) for a period of 10 min. (Moore, 1924, 1951). A similar effect can be produced in rams; semen collected from such animals a week or two later contains only a small number of spermatozoa, mostly dead or degenerate; the seminal plasma on the other hand, retains its normal composition or shows even a slightly elevated content of fructose (Glover, 1954). In bulls, heat-induced azoospermia is said to be associated with an increased excretion of neutral steroids in the urine (Meschaks, 1953). Cold, like heat, has an adverse effect on sperm cells *in vivo*. An ice-pack applied for 10 min. to the testes of a rabbit invariably results in disintegration of spermatozoa in the epididymides (Chang, 1943).

No less dramatic but different in kind are the *in vitro* effects of heat and cold on spermatozoa. Dog and rabbit spermatozoa although capable of survival for several hours *in vitro* at 40°, soon lose their

motility at 45° (Amantea and Krzyszkowsky, 1921; Walton, 1930). Cooling to a temperature just above 0° is not harmful to ejaculated semen *in vitro*, provided however, that the temperature of the ejaculate has been lowered gradually, preferably by successive stages of 5°, with an interval of 2 hr. or so, between each. Sudden cooling of ejaculated semen produces so-called *temperature shock* and involves rapid and irreversible loss of motility and fertilizing power (Gladcinova, 1937; Chang and Walton, 1940; Easley, Mayer and Bogart, 1942). The decline of respiration and fructolysis in 'temperature-shocked' samples of bull semen was shown to be correlated with a proportionate increase of dead, that is eosin-staining, sperm cells (Hancock, 1952).

Provided that strict precautions are observed, semen can be cooled well below 0° without destroying the sperm. Some of the earliest observations concerning the effect of low temperature on sperm (human, bull, stallion, and frog) were made by Spallanzani (1776, 1799). On subjecting stallion spermatozoa 'to the cold of freezing, by putting the glass in which they were, among snow' he made the following observations:

'The same effect was produced by snow, as by the winter's cold; that is, in fourteen minutes, it made the spermatozoa motionless; although when exposed to the heat of the atmosphere, they continued to move seven hours and a half. But an accident that happened in this experiment, executed during summer, afforded new intelligence, and divested me of a prejudice. Observing that the vermiculi had become motionless, I took the glass from the snow, and left it exposed to the air, when the heat was 27°. An hour after, by chance observing this semen with the microscope, I was astonished to find all the vermiculi reanimated, and in such a manner, as if they had just come from the seminal vessels. I then saw, that the cold had not killed them, but had reduced them to a state of complete inaction. I replaced them in the snow, and in three quarters of an hour took them away. These are the phenomena I observed. In a few minutes, their vivacity relaxed, and the diminution increased, until they lost the progressive motion, and retained only that of oscillation, which likewise ended in a few minutes more. Exactly the reverse was observed, when they passed from the cold of the snow to the heat of the atmosphere. The first motion that appeared, was that of oscillation; the body and the tail begun to vibrate languidly

from right to left; then the motion was communicated to the whole vermicule; and, in a short time, the progressive motion begun.'

In the XIXth century, Prévost (1840), de Quatrefages (1853), Mantegazza (1866), Schenk (1870) and others, experimented with sperm exposed to temperatures ranging from 0° to −17°, but it was not until 1938 when Jahnel proved that human spermatozoa can resist the temperature of solid carbon dioxide (−79°), and Luyet and Hodapp demonstrated that frog spermatozoa can survive the temperature of liquid air (−192°), provided that they are mixed with a concentrated solution of sucrose before immersion in liquid air.

These and subsequent studies by other authors, including Shettles (1940), Shaffner (1942), Hoagland and Pincus (1942) and Parkes (1945) have provided further strong support for the general conclusion, elaborated in detail by Luyet and Gehenio (1940) in their treatise on *Life and Death at Low Temperatures*, namely that spermatozoa, not unlike certain bacteria and some flagellates, are remarkably resistant to low temperatures and on vitrification pass into a reversible condition of complete inactivity and quiescence. This was described by Becquerel as 'la vie latente' and has been compared to the behaviour of a watch which, though well wound, can be brought to a sudden standstill by some braking mechanism; such a watch will start of its own accord as soon as the brake is removed. The main principle underlying Luyet's thesis is that cells such as spermatozoa manage to survive at low temperatures if cooling is effected so as to by-pass the crystallization zone, by carrying the cells straight into the range of sub-freezing temperatures known as the vitrification zone, where they assume the non-crystalline, glass-like, or vitreous state. The passage on thawing from the vitreous state equally deserves attention and is best achieved by rapid warming, again to avoid the crystallization zone. When these precautions are maintained, it is possible to prevent colloidal changes ordinarily associated with freezing and ice-crystal formation, such as denaturation and coagulation of proteins, protoplasmic precipitation, release of enzymes and structural disarrangement. In the opinion of Becquerel (1936, 1938), the principal danger to the 'latent life' of cells at low temperatures is the damage to cellular structure which occurs in the freezing zone, caused by the separation of water

and electrolytes from the colloidal particles. This damage can be substantially reduced if freezing is carried out in the presence of certain organic substances such as sucrose, glucose, fructose, glycerol, ethylene glycol, gelatin, albumin and various gums, all of which have been used extensively in the past in freezing and freeze-drying experiments on bacteria, yeasts, protozoa and various other cells and tissues. The application of these substances, however, to sperm is comparatively recent. In 1938, Luyet and Hodapp observed that frog spermatozoa fail to survive the temperature of liquid air, but if the cooling is carried out in the presence of 40% sucrose at least 20% of them revive. Shaffner, Henderson and Card (1941) were able to keep alive 30% of fowl spermatozoa by freezing them to −79°, after treatment with fructose. An attempt to use glycerol in connection with survival experiments on frog spermatozoa was made by Rostand (1946) but it was not until 1949 when the remarkable properties of glycerol were brought into prominence thanks to Polge, Smith and Parkes (1949) as a result of their studies on the low temperature resistance of glycerol-treated fowl semen. In fowl semen diluted with an equal volume of Ringer solution and vitrified at −79° for 20 min. and then rapidly thawed, there was no significant revival of spermatozoa. On the other hand, when the dilution was carried out with Ringer solution containing 40% glycerol, the spermatozoa resumed full motility on thawing. This observation was soon extended to the semen of other animals, including the bull (Smith and Polge, 1950). A large number of cows have been inseminated by Polge and Rowson (1952) with glycerol-treated bull semen which had been stored at −79° for periods of many months, and the excellent fertilizing capacity of such 'deep-frozen' semen was proved when pregnancy occurred in 66% of the inseminated animals. Glycerol-frozen and thawed human semen has been reported to contain motile and fertile spermatozoa (Sherman and Bunge, 1953; Bunge and Sherman, 1953).

As yet, there is no adequate explanation for the effect of glycerol on semen. The suggestion that it acts by supporting some sort of residual metabolism in the frozen spermatozoa is difficult to reconcile with our own observation that glycerol, unlike sugars and fatty acids, is not oxidized by bull or ram spermatozoa. More probably, glycerol exerts a protective influence on spermatozoa, preventing denaturation

changes during freezing. Glycerol has long been recognized in protein and enzyme chemistry as a convenient, 'stabilizing' agent which combines the properties of a protein solvent with the ability to protect the protein from denaturation caused by temperature changes. It has been shown to prevent the heat coagulation of serum and egg albumin (Beilinsson, 1929), and is in use in the cold storage of egg yolk owing to its solubilizing action on lipoproteins (cf. McFarlane and Hall, 1943). Lavin, Northrop and Taylor (1935) used glycerol in their study of pepsin at −100°; Keilin and Hartree (1949) discovered that the presence of glycerol at very low temperatures intensifies the absorption spectra of haemoproteins some twenty-five times. This in turn, made it possible to demonstrate for the first time, the spectrum of cytochrome in human spermatozoa (Mann, 1951*a*). The protecting influence of glycerol on sperm colloids may well be linked with the electrolyte- and water-binding properties of this substance. In this connection, an observation by Luyet deserves to be mentioned, on the existence of a definite relationship between the water-binding capacity of different solutes used for vitrification, and the temperature at which devitrification takes place on thawing; the devitrification temperature of glycerol is in the neighbourhood of −70° (Miner and Dalton, 1953) which is below that of sucrose, fructose, gelatin, various gums and most other solutes.

Role of hormones

Among factors which influence the production of semen in man and animals, hormones rank paramount in importance. The formation, output, and composition of ejaculated semen are the outcome of a concerted action of several endocrine organs, with the pituitary gland and the testicular interstitial tissue in dominant positions.

Apart from the direct gonadotrophic activity due to the gametogenic and interstitial-cell stimulating hormones, the anterior lobe of the pituitary gland exerts an indirect influence upon the male reproductive organs, through interaction with the thyroid gland and the adrenal cortex. The anterior hypophysis itself depends on stimuli from the central nervous system and responds in a particularly sensitive manner to impulses transmitted through the optic nerves. The seasonal fluctuations in the intensity of light impulses, relayed

through the optic nerves, are probably responsible for the changes associated with the so-called male sex cycle; many lower vertebrates produce spermatozoa only during a brief period, once a year, and even among mammals, there are many species in which male sexual activity is restricted to definite season. Sperm survival in the epididymis and sperm transport in the female reproductive tract, are also two phenomena which probably depend on pituitary function; the former on the normal gonadotrophic activity of the anterior lobe, the latter on impulses transmitted to the uterus by the oxytocic hormone secreted in the posterior lobe.

The function of testicular hormone, in so far as semen is concerned, is to provide the stimulus necessary for the elaboration of seminal plasma and for ejaculation. The male sex hormone is intrinsically linked with the production of seminal plasma by the accessory organs; it regulates the secretory activity of the accessory organs, and thus determines not only the output of the seminal plasma as a whole, but also the relative contribution of each individual gland towards the ultimate make-up of semen.

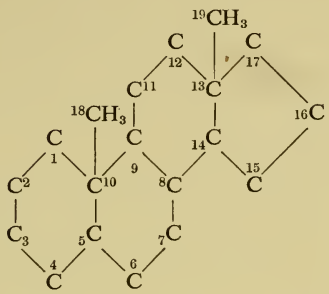
The earlier morphological studies have provided much fundamental information which has helped to build up our knowledge of the relationship between the functional state of the male accessory organs and androgenic activity. These investigations have shown that postcastrate retrogressive changes in the gross appearance and in the microscopic structure of the accessory organs can be prevented, or reversed, by the administration of testicular hormone. On this basis several so-called 'hormone indicator tests' have been elaborated for the detection of male sex hormone activity (cf. Moore, 1937; Price, 1947; Dorfman, 1950). Two such tests also involved semen examination: the so-called 'clotting test' was based on the observation that the formation of a clot in the electrically-induced seminal discharge of a guinea-pig depends on the presence of male sex hormone, and the 'spermatozoon motility test' was derived from the observation that in the epididymis severed from the testis, spermatozoa survive longer if the testis is not removed from the body; presumably, the testicular hormone is capable of stimulating the epididymal cells to secrete some substance necessary for the preservation of epididymal spermatozoa (Moore, 1935; Parsons, 1950).

Of recent years the introduction of biochemical methods made it practicable to follow up and to assess androgenic or gonadotrophic activity, or changes due to hormonal deficiency, by means of quantitative chemical analysis of the seminal plasma. Determinations of fructose, citric acid and phosphatase activity in semen provide excellent evidence of the functional state of the male accessory organs of reproduction. These methods which will be discussed in detail later, are particularly useful in studies of progressive hormonal deficiency, such as is brought about for instance, by defective nutrition. The great advantage of the chemical approach is that it enables the assay of accessory gland function to be carried out in *live* animals, at selected intervals, and over long periods of time.

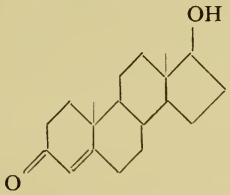
So far, there has been little progress in investigations concerning the influence of hormones on semen *in vitro*. Several hormones have been variously credited with beneficial effects upon the survival, motility and metabolism of sperm *in vitro* (cf. Tschumi, 1946), but in actual fact, apart from isolated observations such as those concerning the stimulating effect of thyroxine on spermatozoa (Carter, 1931, 1932; Lardy and Phillips, 1943*d*; Schultze and Davis, 1948, 1949; Maqsood, 1952), the evidence at hand requires much strengthening before the various claims are accepted as valid. The same is true of studies on the content of hormones in semen itself. There are indications that semen contains some oestrogenic substances (Green-Armytage *et al.*, 1947; McCullagh and Schaffenburg, 1951; Mukherjee *et al.*, 1951) which is not improbable since oestrogenic hormones occur elsewhere in the male body, notably in the testis and in the urine. Diczfalusy (1954) examined by countercurrent distribution and fluorimetric analysis an alcoholic extract from a litre of human semen and found in this material 10 μg. of oestradiol-17β, 30 μg. oestriol, and 60 μg. oestrone, all in a free non-conjugated form.

With respect to androgens, the Dirscherl-Zilliken colour reaction for dehydro*iso*androsterone is strongly positive in extracts from hydrolysed spermatozoa. According to Dirscherl and Knüchel (1950), the content of the colour-yielding material corresponds to about 5·5 mg. 'dehydroandrosterone' in 100 ml. human semen, as compared with 0·1 mg./100 ml. in human urine; for bull and stallion semen the values are given as 4·3 and 1·6 mg./100 ml., respectively.

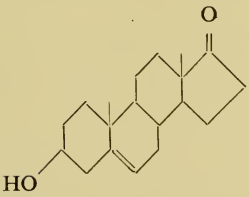
The bulk of the androgenic material appears to occur in a bound form and can be set free by hydrolysis with hydrochloric acid.



The tetracyclic carbon skeleton in androgens



Testosterone
(Δ^4-androsten-17-ol-3-one)



Dehydro*iso*androsterone
(Δ^5-androstan-3(β)-ol-17-one)

Sperm-egg interacting substances and chemotaxis

The so-called sperm-egg interacting substances comprise a group of agents which have received considerable attention from pioneers in the field of sex physiology such as Lillie (1919) and Loeb (1913),

but have acquired even more prominence and a wider significance in recent times, chiefly as the outcome of investigations by the schools of Hartmann in Germany, Runnström in Sweden, and Tyler in the United States. So far, research on these substances has been limited largely to invertebrates; it has been thoroughly reviewed by Tyler (1948), Bielig and von Medem (1949), Runnström (1949, 1951), Brachet (1950) and Rothschild (1951*a*, *b*) and will therefore be mentioned here only briefly.

The fundamental observations on sperm-egg interacting substances were made by Frank Lillie who discovered that the so-called 'egg-water', that is sea-water with which sea-urchin eggs have remained in contact for a while, is enriched with some substances derived from the eggs, and capable of inducing the agglutination of homologous spermatozoa. Lillie was convinced that the sperm-agglutinating agent plays a significant role in fertilization and called it 'fertilizin'. Another effect produced by egg-water, which he noted in certain species of marine animals, was an increase in the motility of spermatozoa. This phenomenon he also ascribed to 'fertilizin'; the substance in the sperm, with which fertilizin was believed to combine, was named 'antifertilizin'.

The sperm-egg interacting substances, mostly studied in sea-urchins, are sometimes referred to as 'gamones'; those derived from eggs being called 'gynogamones', as opposed to 'androgamones' in sperm.

Gynogamone I is the name given to the agent responsible for the activating influence of egg-water on spermatozoa. Hartmann, Schartau, Kuhn and Wallenfels (1939) thought that in the case of *Arbacia pustulosa* the sperm-activating substance is chemically related to echinochrome, the pigment discovered in 1885 by MacMunn. They found that pure echinochrome A (2-ethyl-3 : 5 : 6 : 7 : 8-pentahydroxynaphtoquinone) isolated from ripe eggs of *Arbacia pustulosa*, exerts a stimulating effect on the movements of sea-urchin sperm, even in a 1 : 2,500,000,000 dilution. This claim, however, has been seriously challenged by Tyler (1939) and Cornman (1940, 1941).

At this point it may be relevant to recall two observations, one by Clowes and Bachman (1921*a*, *b*) who noted that a sperm-stimulating agent can be separated from egg-water by distillation, and the other a finding made by Carter (1931) that the

activation effect of egg-water on spermatozoa can be reproduced by thyroxine.

Gynogamone II, also called 'isoagglutinin', is the name applied to the fertilizin responsible for the agglutinating action of egg-water upon spermatozoa of the same species. This substance originates in the gelatinous material or 'jelly coat' surrounding the eggs, and passes therefrom into the sea-water; chemically it is a mucoprotein which differs in composition according to species; the polysaccharide component has been reported to contain sulphuric acid in addition to galactose, fucose, glucose, or fructose (Vasseur, 1947, 1949; Tyler, 1948; Bishop and Metz, 1952).

The group of androgamones comprises three substances. Androgamone I, to which further reference will be made later in connection with its alleged role as a sperm-immobilizing agent, antagonizes the action of gynogamone I (Hartmann, Schartau and Wallenfels, 1940). It is a heat-stable, alcohol-soluble factor which can be extracted from sea-urchin spermatozoa by sharp centrifugation or with methanol, but it is still uncertain whether or not it actually diffuses out of intact sperm cells. Androgamone II is the antifertilizin which reacts with the sperm-agglutinating gynagamone II. It is an alcohol-insoluble, protein-like substance extracted by Hartmann and his associates from sea-urchin sperm, and believed to function as a jelly-coat dissolving or precipitating factor. Androgamone III, also known as the 'egg-surface liquefying agent' or 'lysin', is an alcohol-soluble substance found by Runnström, Lindvall and Tiselius (1944) in sea-urchin and salmon spermatozoa, with a lytic action towards the cortical layer of eggs. It is probably a fatty acid; in its effect on sea-urchin eggs it resembles bee venom and certain detergents (Runnström and Lindvall, 1946).

Much confusion in the past has been caused by conflicting reports which ascribed to gynogamones, apart from their activating or agglutinating action, also a definite attracting or chemotactic influence on spermatozoa. It is doubtful whether in animals chemotaxis plays any serious role in guiding the spermatozoa to the eggs. Plants, on the other hand, provide several excellent examples for the existence of chemotaxis (Cook, 1945; Hawker, 1951). In mosses and ferns, the spermatozoids are well known to be attracted towards various substances, for example malic acid, sucrose, certain salts

and alkaloids; in a few instances, some of these substances have actually been claimed to occur in the archegonia (Pfeffer, 1884; Shibata, 1911). Bracken spermatozoa are attracted by the *cis* but not the *trans*, configuration of organic acids; thus, they can be shown to move towards maleic but not fumaric, and towards citraconic but not mesaconic, acid (Rothschild, 1952). Several organic compounds, including some simple hydrocarbons, ethers and esters, have been shown to possess chemotropic activity for the sperm of certain *Fucaceae* (Cook, Elvidge and Heilbron, 1948; Cook and Elvidge, 1951).

Cross-pollination between two flowering plants of *Forsythia* is brought about by an exchange, followed by enzymic breakdown, of two glycosides of the natural flavonol pigment quercetin, carried with the pollen, namely rutin (quercetin rutinoside) and quercitrin (quercetin rhamnoside) (Kuhn and Löw, 1949; Moewus, 1950). Plants also provide several instances of differential distribution of pigments in the male and female gametes. Among the fungi, the small motile male gamete of some species of *Allomyces* is distinguished from the larger female gamete by the presence of an orange-coloured globule in which Emerson and Fox (1940) have found γ-carotene along with traces of isomers. In the family *Fucaceae*, chemical resolution of the pigments from the male and female exudates of several species has shown that the predominant colouring matter of the orange-coloured male gametes consists of β-carotene, whereas the olive-green pigmented eggs contain chlorophyll and fucoxanthin (Carter, Cross, Heilbron and Jones, 1948). The participation of carotenoids in the reproduction of algae is indicated by studies on the unicellular flagellate alga, *Chlamydomonas*, where picrocrocin, crocin, and *cis-trans*-crocetin dimethyl esters have been shown to play a role in the conjugation of the male and female gametes as well as in sex determination (Kuhn, Moewus and Jerchel, 1938; Kuhn and Moewus, 1940).

In animals, the position of carotenoids as sperm-active substances remains uncertain, but there is the significant fact that remarkably large amounts of carotenoid pigments occur in male gonads and accessory glands of reproduction of many animals, including mammals (Goodwin, 1950). The importance of vitamin A and carotene in developing and maintaining the normal germinal

epithelium in bulls has been stressed repeatedly by many investigators (cf. Bratton *et al.*, 1948).

'Dilution effect' and chemical changes associated with sperm senescence

The changes which result from dilution of semen have been the subject of much study; two distinct lines of research are recognizable. The chief endeavour of the mammalian semen investigators was to solve the practical problem of the composition of artificial diluents, whereas the workers interested in the sperm of lower forms, such as the sea-urchin, were trying to establish the cause, rather than the remedy, for the effect of dilution. It may be said at once that as yet, there is no perfect semen diluent, and the precise mechanism of the 'dilution effect' still remains to be solved but much progress has been made in both directions.

Some of the early experiments on semen dilution, by Koelliker (1856), Ankermann (1857), Engelmann (1868), and others, were carried out with frog spermatozoa; in semen pressed out directly from the frog testis, the spermatozoa were found to be motionless but when mixed with a few parts of water, they became intensely motile. This activity, however, was of short duration; it began to decline already after a few minutes and seldom extended beyond one hour. Prolonged motility was obtained when water was replaced with 0·25 to 0·5% NaCl solutions. In the presence of higher concentrations of NaCl the spermatozoa remained motionless but could be 'revived', even after a relatively long time, by further addition of water. It was also shown that the presence of oxygen is not absolutely essential for the motility of frog spermatozoa. This was first demonstrated in 1868 by Engelmann at Utrecht who found that frog sperm motility could be maintained for several hours in diluted semen, in an atmosphere of hydrogen or carbon monoxide; however, when this 'anaerobic' motility began to decline, it was restored by pure oxygen or air.

The response of fish and sea-urchin spermatozoa to dilution with water or salt solutions is not very different from that of frog sperm. The addition of water or dilute salt solutions to trout semen provokes a shortlived burst of activity followed by gradual exhaustion and death of the spermatozoa; oxygen has an activating effect on

the motility and prolongs the life of the spermatozoa (Scheuring, 1928). Trout spermatozoa have been a favourite object for investigations on the action of various cations and anions on sperm motility (Scheuring, 1928; Gaschott, 1928; Schlenk, 1933; Schlenk and Kahmann, 1938). Among the many facts brought to light by these investigations, the effect of potassium ions merits particular attention. In contrast to sodium chloride, a diluent containing 0·15% potassium chloride was found to have no activating effect on sperm motility, and moreover, the addition of potassium ions to a suspension of motile sperm in sodium chloride, rendered them motionless. This inhibition by potassium ions, however, was shown to be reversible since even after prolonged storage of sperm in the presence of potassium ions, it was still possible to restore their motility by dilution with water or sodium chloride solutions. The fact that trout spermatozoa show great activity upon dilution with water or sodium chloride solution but not with trout seminal plasma, has been attributed by Schlenk to the high potassium content of the latter (80 mg./100 ml.); in his view, the rapid increase of motility after dilution with water should be ascribed to the decrease of potassium concentration in the seminal plasma and the passage of potassium ions from the sperm cells into the surrounding medium. According to this interpretation, potassium ions fulfil a double function in semen: they preserve the sperm energy by inducing quiescence, but at the same time, they engender, as it were, a state of preparedness ('Bewegungsbereitschaft').

Much information on the effect of dilution has been gathered from experiments with sea-urchin semen. The spermatozoa of sea-urchins, unlike those of man and higher animals, are immotile in the absence of oxygen (Harvey, 1930; Barron, 1932). In sea-urchin semen which generally has a high sperm density (there are some 2×10^{10} cells/ml. in *Echinus esculentus*), the spermatozoa are motionless so long as they remain undiluted, but when shed into or artificially diluted with, sea-water, they become intensely motile and with increasing dilution their oxygen uptake rises as well (Gray, 1928, 1931). The lack of sperm movement in undiluted sea-urchin semen has been regarded by Gray as the outcome of mechanical overcrowding; each cell exercising a restraining or allelostatic effect on the activity of its neighbours. Other authors believed the lack

of movement in undiluted semen to be due not so much to mutual restraint, as to the presence of a specific sperm-immobilizing substance either in the sperm or in the seminal plasma. A suggestion has been put forward that the main cause for the lack of motility is due to the sperm-paralysing influence of androgamone I. Another line of thought was that sperm quiescence is due to the seminal plasma acting by virtue of its high potassium content or, alternately, its low hydrogen ion concentration.

The various theories concerned with the lack of sperm movement in undiluted semen have been reviewed and analysed by Rothschild (1948*c*, 1950*d*, 1951*a*, *b*). There is, he argues, no conclusive evidence that the spermatozoa remain motionless in undiluted sea-urchin semen because of any one factor such as allelostasis, sperm-immobilization, pH, carbon dioxide or potassium ions. It is particularly difficult, he points out, to reconcile the observations on the immobilizing action of seminal plasma with the fact that seminal plasma obtained by gentle centrifugation and used as a diluent for fresh semen, renders the spermatozoa as active as sea-water. He found that the seminal plasma of *Echinus esculentus* acquires sperm-immobilizing properties only after prolonged centrifugation of semen, presumably as the result of leakage of an inhibitory substance from the cells; the rate at which the immobilizing substance diffuses from the spermatozoa into the seminal plasma seems to vary and this probably explains the conflicting reports concerning the effect of centrifuged seminal plasma on sperm motility. Rothschild's own experiments indicate that the main cause for the lack of sperm movement in undiluted sea-urchin semen is deficiency of oxygen; in fact, spermatozoa can be mobilized even in undiluted semen by increased oxygen tension, and deprived of motility through oxygen withdrawal. According to this author, the sudden outburst of activity upon dilution of semen, is simply due to an improved access of the spermatozoa to oxygen.

As in frog and fish, the marked motility evoked in sea-urchin sperm by dilution is only of limited duration and is subject to progressive decline in spite of the presence of oxygen. If the dilution with sea-water is very excessive, the decline may almost immediately follow the onset of activity. Gray (1928, 1931) thought that the progressive loss of activity in dilute sperm suspensions could be

explained by spontaneous and irreversible senile decay due to the gradual disruption of cellular organization, exhaustion of food reserve, depletion of energy, and autointoxication with the reaction products accumulated during the period of activity. He showed that it is possible to delay the decline of activity by replacing the sea-water with egg-water; a similar effect has been later demonstrated with solutions of the egg-surrounding jelly. In this respect, however, egg-water cannot be looked upon as a very specific agent. Both sperm motility and respiration can be extended, for instance, by the addition of seminal plasma, and there are several indications that this is due to proteins and their breakdown products in the seminal plasma. Hayashi (1945, 1946) experimenting with *Arbacia punctulata*, demonstrated the occurrence in the seminal plasma of a non-dialysable constituent beneficial to the viability and fertilizing capacity of spermatozoa. Wicklund (1949, 1952) demonstrated a favourable influence of serum albumin on the fertilizing capacity of sea-urchin spermatozoa; she found that the fertilizing power of washed or aged sperm of *Psammechinus miliaris* was retained much longer following dilution with albumin solutions than with sea-water. Tyler (1950) and Tyler and Atkinson (1950) found that the life-span of sea-urchin sperm can be considerably extended by the addition of certain peptides and amino acids. Tyler and Rothschild (1951) examined the sperm metabolism of *Arbacia punctulata* and *Lytechinus* in sea-water enriched with amino acids and noted that under such experimental conditions the initial increase of respiration characteristic of the 'dilution effect', was less pronounced but the subsequent decline in activity was considerably delayed, and the total amount of oxygen consumed greatly increased. These facts, coupled with evidence of non-utilization of the added amino acids, indicated that the amino acids enabled the spermatozoa to make fuller use of their endogenous substrate, probably by inducing the formation of complexes with copper and other toxic heavy metals commonly present in sea-water. This hypothesis has gained additional support from the results of further work on the detoxicating effect of metal-chelating agents such as ethylenediamine tetra-acetate (versene), diethyldithiocarbamate, α-benzoinoxime (cupron) and 8-hydroxyquinoline (Tyler, 1953). Perhaps the beneficial action of proteins (Metz, 1945; Wicklund, 1949) and of seminal plasma

(Hayashi, 1945, 1946; Chang, 1949) on spermatozoa is also, partly at any rate, due to similar processes.

The mechanism of inactivation and senescence induced in sea-urchin spermatozoa by prolonged dilution remains obscure but there is no reason to suppose that it differs intrinsically from senescence in mammalian spermatozoa. In the latter, senescence is known to be associated with certain definite chemical and physical changes, such as oxidation of intracellular sulphydryl groups which are essential for normal motility, decrease in the content of adenosine triphosphate, and increased sperm permeability, which leads to the extracellular appearance of intracellular sperm-proteins; it is also probable that an early change in senescence causes swelling or some other degeneration in the lipoprotein-containing 'lipid capsule' which normally surrounds the sperm cell (see p. 126). It is by no means unlikely that some upset in the progress of ionic exchange reactions involving particularly potassium ions, is also linked with senescence.

The effect of dilution on mammalian spermatozoa is essentially the same as in the sperm of lower animals. Dilution with small volumes of saline solution produces 'activation' or excitation, a phenomenon well known to Koelliker and other investigators of the XIXth century. The extent of this activation depends of course, on concomitant factors such as pH, temperature, oxygen, and the presence of certain substances. For example, to obtain optimal motility and metabolism in diluted bull sperm, Lardy and Phillips (1943*a*) advise the addition of at least 0·005M potassium and 0·012M magnesium, with simultaneous omission of calcium ions. The need for potassium ions has been re-emphasized by Blackshaw (1953). The inclusion of phosphate, mainly as a buffer, has been advocated repeatedly by several authors, even though in higher concentrations it depresses motility and respiration of bull sperm (Bishop and Salisbury, 1954). Sulphate ions have been recommended by Milovanov on the ground that they prevent the swelling of spermatozoa and protect the 'lipid capsule' from the action of sodium chloride.

In the writer's laboratory, the following salt solution, similar in composition to the Krebs–Henseleit-Ringer solution, is used routinely as a diluent for ram and bull spermatozoa, 100 ml. 0·9% NaCl, 4 ml. 1·15% KCl, 1 ml. 2·11% KH_2PO_4, 1 ml. 3·82% $MgSO_4 \cdot 7H_2O$, 2 ml. of a 1·3% solution of $NaHCO_3$ saturated with

CO_2. In some experiments this is supplemented with (i) 0·5 g. fructose/100 ml. ('Ringer-fructose'), (ii) 20 ml. 0·25 M-phosphate buffer pH 7·4, the latter made up by mixing 19 ml. of an aqueous solution containing 0·71 g. Na_2HPO_4 (or 1·79 g. $Na_2HPO_4,12H_2O$) with 1 ml. N-HCl ('Ringer-phosphate') or (iii) both fructose and phosphate ('Ringer-fructose-phosphate'). Glass-distilled water and analytical grade reagents are used throughout (Mann, 1946*b*).

If the dilution of mammalian sperm with saline is excessive it leads after a short spell of increased activity to a permanent loss of motility, metabolic activity and fertilizing capacity. According to Milovanov (1934), the resistance (R) of sperm to the immobilizing effect of dilution with large volumes of 1% NaCl, varies in different animals; he believes that specimens of semen capable of high resistance possess at the same time high fertilizing capacity. The formula $R=\frac{V}{v}$ used by Milovanov in the 'resistance test', is calculated from the volume of 1% NaCl (V) required to arrest in a given volume of semen (v) all progressive movement of sperm-heads. In bull semen the test is carried out with 0·02 ml. semen, at 17–24°, and sperm motility is assessed by microscopic examination after the addition of successive lots of 10 ml. 1% NaCl solution (Nagornyi and Smirnov, 1939). The R values given by Milovanov are: bull 300–20,000, ram 100–5000, stallion 100–1500, boar 60–1000, dog 200–600. Some doubts however, have arisen as to the significance of Milovanov's test in the assessment of male fertility, as well as his statement that the immobilizing action is due to the toxicity of sodium chloride as such (Emmens and Swyer, 1947, 1948; Cheng, Casida and Barrett, 1949). But there is general agreement that excessive dilution is invariably harmful to spermatozoa. This is reflected, among others, in Chang's (1946) finding that a constant number of rabbit spermatozoa has a greater fertilizing capacity in a small, as opposed to a large, volume of salt diluent. Chang inseminated superovulated does with saline suspensions of spermatozoa, removed the ova 38–42 hr. later, and counted the cleaved ova. Results showed that the insemination of a total of 30–40 thousand spermatozoa in 1 ml. 0·9% NaCl was followed by the cleavage of 0–6% of ova, whereas the same number of cells suspended in 0·1 ml. produced cleavage in 17–42% of ova. Another observation which

confirms the adverse effect of dilution is, that although the addition of fructose to an isotonic salt diluent prolongs the metabolic activity of bull sperm suspensions, excessive dilution with 'Ringer-fructose' solution leads to a decline in the rate of fructolysis (Mann and Lutwak-Mann, 1948). Up to a point, the changes due to the 'dilution effect' resemble those produced by extensive washing of spermatozoa. Thus for example, whereas one careful washing of centrifuged ram sperm with several volumes of Ringer solution causes but negligible damage, repeated washing results in a progressive decline in motility and metabolism (Mann, 1945*a*; White, 1953).

The use of artificial diluents in the storage of semen

It has been noticed repeatedly that the deleterious effect of salt diluents on spermatozoa can be at least partly overcome by the inclusion of certain organic substances, mainly of colloidal nature. These observations stimulated the development of the so-called 'media', 'pabula', 'dilutors' and 'extenders', for use in the storage of semen for artificial insemination.

The use of diluents in the storage of semen has its origin in certain experiments made by Donné, de Quatrefages, and Koelliker, who examined the effect of blood plasma, milk, various proteins and sugars, on the spermatozoa. Early in this century, Hirokawa (1909), Champy (1913), and others, experimented with blood plasma and serum. Particularly illuminating results were obtained by Grodziński and Marchlewski (1938) who stored cock semen diluted ten times with chicken serum at 2° for periods up to eight days, and found, on increasing the temperature to 37°, that the spermatozoa were motile. In addition, however, these authors also found, in agreement with Bernstein and Lazarev (1933), that it is advisable to use blood serum which has been pre-treated at 55°, as otherwise the spermatozoa tend to agglutinate. An agglutinating and spermicidal factor, present in fresh serum but inactivated by a few days' storage at 4°, or 10 minutes' heating at 56°, was found by Chang (1947*a*) in human, bovine, rabbit, guinea pig, and rat serum. It is mainly because of its agglutinating properties that blood serum has not found a wider application as a semen diluent. On the other hand, as a direct result of immunological studies on the reactions between blood serum and spermatozoa, rapid progress has been

made in studies concerning spermatozoal antigens and antibodies, culminating in the development of several antispermatozoal sera, active not only towards heterologous but also homologous, spermatozoa. So far, most attempts to immunize a female against spermatozoa of her own species, as a means of 'serological contraception', have been failures. A wealth of data on sperm immunology and sperm-serum interaction can be found in articles by Metchnikoff (1900), Moxter (1900), Godlewski (1912), Kato (1936), Henle and Henle (1940), Parkes (1944), Snell (1944), Tyler (1948), Smith (1949), Docton *et al.* (1952), and Kibrick *et al.* (1952).

One of the first diluents used in veterinary practice for the insemination of cattle in Russia, was Milovanov's 'SGC-2 dilutor' (13·6 g. Na_2SO_4, 12 g. glucose, and 5 g. Witte's peptone, in 1 l. water). The same investigator experimented also with so-called gelatinized diluents which contained apart from salts and glucose, some gelatin, so as to endow the fluid with a jelly-like consistency. One such diluent developed for the storage of bull semen, has been the 'GPC-3-G dilutor' (1·7 g. Na_2HPO_4, 0·07g. KH_2PO_4, 0·08 g. Na_2SO_4, 2·85 g. glucose, 5·00 g. gelatin, 1 l. water.) A method adopted by the Russian workers was to use the diluted semen for insemination in the form of gelatin capsules. A major advance in the technique of semen storage has been the introduction by Phillips and Lardy (1939, 1940) of the 'egg-yolk-phosphate diluent' which became widely established both in America and in Europe for the purpose of preservation, transportation and insemination of bull semen. It is prepared by mixing freshly separated egg yolk with an equal volume of phosphate buffer, pH 7·4 (2 g. $Na_2HPO_4 \cdot 12H_2O$, 0·2 g. KH_2PO_4, made up to 100 ml. with water). For actual storage, and subsequent insemination, bull semen is diluted up to 100 times, or more, with the egg-yolk phosphate dilutor. In addition to preserving sperm viability, the dilutor protects the spermatozoa efficiently from 'temperature shock', that is from the rapid immobilization induced by sudden cooling of semen to 5–10°, the usual temperature for storage of semen (Chang and Walton, 1940; Easley, Mayer and Bogart, 1942). The chemical nature of the protecting substance is unknown but there are indications that it is an acetone-soluble but ether-insoluble, compound (Mayer and Lasley, 1944).

Many recommendations have been made to improve the egg-yolk-

buffer diluent by the inclusion of various additives such as gelatine (Knoop, 1941), glycine (Knoop and Krauss, 1944; Tyler and Tanabe, 1952), sodium citrate (Willett and Salisbury, 1942; Salisbury, Knodt and Bratton, 1948), bicarbonate and glucose (Kampschmidt, Mayer, Herman and Dickerson, 1951), dialysed yolk (Tosic and Walton, 1946*a*) and liquid whole egg (Dunn and Bratton, 1950); to counteract the danger from bacterial contamination, certain antibiotics are sometimes added such as penicillin, streptomycin, polymixin, aureomycin or sulphonamide drugs. On this subject alone there is a vast number of publications of which only a few can be quoted here (Knodt and Salisbury, 1946; Bayley, Cobbs and Barrett, 1950; Branton, James, Patrick and Newsom, 1951; Foote and Bratton, 1949, 1950; Hennaux, Dimitropoulos and Cordiez, 1947; Pursley and Herman, 1950; VanDemark, Salisbury and Bratton, 1949; VanDemark, Bratton and Foote, 1950; Willett, 1950; Dunn, Bratton and Henderson, 1953).

Some attempts were made a while ago to replace the egg-yolk buffer diluent by a chemically more clearly defined, artificial medium. Thus, Phillips and Spitzer (1946) developed the so-called 'L.G.B.-pabulum' which contained as essential ingredients 1–2% of a lipid fraction (L), made up of lipositol (an inositol-containing phospholipid), 0·6% glucose (G), phosphate buffer (B), pH 7·4 (0·2% KH_2PO_4 and 2% $Na_2HPO_4 \cdot 12H_2O$), with 0·2% galactose, 0·03% of sulphasuxidine or streptomycin, and lastly 3% gum acacia, added to provide 'sufficient body to prevent the settling out of sperm upon standing in storage'. Other substitutes for egg-yolk which have been suggested at various times, include milk, glycerol, paraffin, arachis oil and synthetic plasma-substitutes such as 'periston' (Laplaud, Bruneel and Galland, 1951; Koch and Robillard, 1945; Rostand, 1946, 1952; Asher and Kaemmerer, 1950; Thacker and Almquist, 1953).

It remains for future investigations to invent an ideal diluent. Such a diluent would be expected to combine the following features, isotonicity, efficient buffering capacity, nutrient value, antibacterial potency, stabilizing action of a 'protective colloid', anti-oxidant ability, and above all, good keeping quality in a ready-to-use form. Furthermore, it should protect semen from the effects of sudden changes of temperature and preserve its full fertilizing capacity for a

reasonable period at the low temperatures of storage *in vitro*. There is also the problem as to whether semen should be stored in a diluted form or whether it would be better to dilute it just before actual use. It would appear that on the whole, under natural *in vivo* conditions, spermatozoa survive best in a highly concentrated state when their motility is reduced to a minimum; the prolonged life-span of sperm in the epididymis certainly points in that direction. Another even more suggestive example is the behaviour of ejaculated bat sperm: the density of sperm as found in the bat uterus after copulation is very high, about 6 million cells/μl. (Schwab, 1952); in this condition the spermatozoa seem to be largely immotile, but are nevertheless capable of survival for several months. Such spermatozoa respond to artificial dilution by becoming intensely motile, but then they survive for not longer than a few days.

CHAPTER IV

Intracellular Enzymes, Metalloproteins, Nucleoproteins, and other Protein Constituents of Spermatozoa

Mechanical separation of sperm from seminal plasma; release of intracellular proteins from damaged spermatozoa. Removal of the sperm nucleus from the cytoplasm. Protein-bound iron, zinc, and copper. Cytochrome. Catalase. Hyaluronidase and other 'lytic' agents. Sperm nucleoproteins. Deoxyribonucleic acid. The basic nuclear proteins; protamines and histones. The non-basic nuclear proteins; karyogen and chromosomin. Keratin-like protein of the sperm membrane.

Mechanical separation of sperm from seminal plasma; release of intracellular proteins from damaged spermatozoa

A STUDY of the proteins present in the spermatozoa themselves calls for an efficient separation of the sperm from the seminal plasma, by centrifugation and washing. However, the spermatozoa are filiform structures, highly vulnerable to mechanical damage. Centrifugation, dilution and washing may inflict an injury upon the sperm cell which, even if not apparent upon ordinary microscopic examination, nevertheless results in a leakage of certain proteins from the spermatozoa into the surrounding medium. Thus, for instance, cytochrome *c* is easily detached from the sperm structure as a result of cellular damage or prolonged storage of spermatozoa (Mann, 1951*a*); because of that, the spectroscopic detection of extracellular cytochrome *c* provides a sensitive indicator of 'senescence' changes in spermatozoa. Another example is the release of hyaluronidase by the spermatozoa (see p. 94). At one time, this phenomenon was ascribed to a true secretory function of the normal sperm cells but more recent evidence suggests that the liberation of hyaluronidase takes place in an ageing or moribund cell population. Yet another phenomenon in this category is the loss of lipoprotein from the 'lipid capsule' of the sperm cell which may easily occur as

a result of extensive washing. However, even if the separation of sperm from the seminal plasma has been carried out with due care and attention, there is no certainty that a loss of intracellular protein has not been incurred. For this reason, one cannot but view with suspicion the results of protein analyses in sperm, if they have been performed with spermatozoa centrifuged at high speed, or washed extensively with large volumes of diluents, some of them anisotonic or unbuffered.

The data at present available indicate that spermatozoa have a much higher concentration of proteins than the seminal plasma. Friedrich Miescher (1870, 1878, 1897) whose fundamental studies provided the earliest information on the chemical nature of some of the sperm proteins, was also the first to point out that in salmon, for instance, the high dry weight and protein content of semen was almost entirely due to the spermatozoa, whereas the seminal plasma gave practically no precipitate with 2 vol. of acidified ethanol, and contained no more than 0·78% dry matter, of this 0·65% mineral, and only 0·13% organic, material. However, a more recent analysis of *Salmo fontinalis* has shown a content of 1·76% nitrogen and 0·43% phosphorus in the seminal plasma (Felix, Fischer, Krekels and Mohr, 1951). Sea-urchin (*Arbacia punctulata*) seminal plasma has about 0·25% protein (Hayashi, 1945).

TABLE 10. *Protein composition of bull semen* (Sarkar *et al.*, 1947)

	In dried material, ash and lipid-free (mg./100 g.)	
	Spermatozoa	Seminal plasma
Total nitrogen	17·61	12·05
Arginine	25·47	7·91
Histidine	2·54	2·13
Lysine	5·08	4·86
Tryptophan	1·59	2·63
Phenylalanine	3·81	3·42
Methionine	1·81	1·61
Threonine	3·78	3·20
Leucine	5·20	3·81
Isoleucine	3·42	2·79
Valine	3·73	3·11
Glutamic acid	8·33	7·75

On the mammalian side, bull semen has received much attention from protein analysts. Zittle and O'Dell (1941*a*, *b*) investigated the nature of the sulphur in bull sperm and found that over two-thirds of the 1·6% S present in lipid-free dry material is accounted for by cystine and cysteine, and the remainder by methionine. Sarkar, Luecke and Duncan (1947) whose results are shown in Table 10, analysed separately frozen-dried bull spermatozoa (20 g. dry material from 100 g. fresh washed sperm) and seminal plasma (1·4 g. dry material from 100 ml.) for total nitrogen and amino acid content. The amino acids were assayed by microbiological methods in protein hydrolysates; however, with the exception of arginine, and to a small extent leucine and tryptophan, the result of the amino acid analysis failed to reflect the different physical character and physiological function of proteins in spermatozoa and seminal plasma. The conspicuously high content of arginine in the spermatozoa is, of course, due to the presence of this amino acid in the nucleoprotein, but even in the seminal plasma the proportion of arginine exceeds considerably that of any other amino acid, with the possible exception of glutamic acid. Further data on the composition of bull sperm protein have been presented by Porter, Shankman and Melampy (1951) who found in extensively washed, lipid-free and dried spermatozoa 16·7% nitrogen; in addition to the amino acids recorded previously, they identified aspartic acid (5%), glycine (1·7%), proline (3·1%), serine (4·5%) and tyrosine (4·3%).

There is but little information apart from some immunological studies, on the chemical differences between the sperm proteins of various species. An early attempt in this direction was made by Fauré-Fremiet (1913) who purified 'ascaridine', a protein peculiar to the testicular tissue, and probably also to spermatozoa, of *Ascaris megalocephala*; an interesing account of this and other unusual characteristics of *Ascaris* sperm is given by Panijel (1951).

Removal of the sperm nucleus from the cytoplasm

Special techniques are required to sever the sperm-head from the tail, as a preliminary to protein analysis in these two morphological components of the sperm cell. Miescher, who pioneered in this field, selected for his studies fish spermatozoa where a separation can be accomplished relatively easily with water or dilute organic acids,

which plasmolyse the tails (together with the middle-pieces), but not the heads. In this way he obtained by centrifugation two portions, a supernatant fluid representing the cytoplasm, and a deposit consisting of sperm-heads which could be further purified by washing with water.

According to Miescher's calculations, in salmon spermatozoa the heads and tails contribute 76 and 24% of fresh material, and 87 and 13% of the lipid-free material, respectively. Suspensions of fish sperm-heads obtained by plasmolysis, centrifugation and washing, are largely sperm nuclei and that is why they have been used extensively for the study of nucleoproteins by Miescher and others who followed in his footsteps. It is, however, rather uncertain what proportion of cytoplasm defies aqueous extraction and how much protein is lost from the sperm-heads in the course of washing. It is quite likely that losses of varying magnitude occur, which would account for the discrepancies in analytical results obtained by different authors, particularly as regards the content of cytoplasmic and non-basic nuclear proteins of fish spermatozoa.

The supernatant fluid obtained by centrifugation of plasmolysed salmon spermatozoa was found by Miescher to be rich in soluble proteins and lipids. On addition of ethanol he obtained two fractions, one which was ethanol-insoluble, accounted for 41·9% of dry material and contained mainly protein (C 51·85, H 7·10, N 14·94, S 1·37), and the other ethanol-soluble, equal to 51·8% of dry material and made up of lecithin, fat and cholesterol. Salmon sperm cytoplasm is known to contain phosphatases active, amongst others, towards adenosine triphosphate; it is devoid of deoxynucleoproteins but contains apparently some ribonucleic acid and several free amino acids, namely alanine, valine, isoleucine, tyrosine, aspartic acid, and glutamic acid (Felix *et al.*, 1951).

Mammalian spermatozoa, in contrast to those of fishes, cannot be plasmolysed, and their heads do not come off in water or acid. To overcome this obstacle, Zittle and O'Dell (1941*a*, *b*) exposed bovine epididymal spermatozoa to ultrasonic waves and in this way dissociated the sperm-heads from the middle-pieces and tails. On slow centrifugation of the disintegrated sperm suspensions, the heads settled out first; at increasing speed, the middle-pieces also formed a sediment, leaving in the supernatant fluid most of the fragmented

tails. The products thus obtained were extracted with lipid solvents and dried. The lipid-free dry weights of heads, middle-pieces and tails were 51, 16, and 33%, respectively, of the whole spermatozoa. The content of ash, nitrogen, phosphorus, sulphur, cystine, and methionine in the three fractions is shown in Table 11.

TABLE 11. *Composition of sperm-heads, middle-pieces, and tails, dissociated by ultrasonic disintegration of bull spermatozoa* (Zittle and O'Dell, 1941*a*)

	In dried, lipid-free material (%)					
	Ash	Nitrogen	Phosphorus	Sulphur		
				Total	Cystine	Methionine
Whole sperm cells	1·8	16·4	2·7	1·6	1·09	0·38
Heads	2·1	18·5	4·0*	1·6	1·07	0·22
Middle-pieces	1·1	16·0	1·6	1·8	1·18	0·48
Tails	1·1	13·6	0·5	1·5	0·88	0·88

* Corresponding to a content of 40·5% deoxyribonucleic acid.

In the author's experience (Mann, 1949, 1951*b*), a relatively simple procedure for the disruption of ram spermatozoa is to shake them with fine glass beads in the mechanical disintegrator of Mickle (1948). Such treatment leads to fragmentation of the middle-pieces and tails, though not of the sperm-heads, and yields on slow centrifugation a yellow-coloured, opalescent fluid which probably represents the sperm cytoplasm. This material is very rich in enzymes; it contains among others, the intermediary enzymes of fructolysis, certain phosphatases, and the complete cytochrome-cytochrome oxidase system, as well as a potent succinic dehydrogenase, the activity of which can be demonstrated both by methylene-blue reduction and by oxygen uptake in presence of succinate. The succinic dehydrogenase activity shown by disintegrated ram spermatozoa contrasts strikingly with the behaviour of the fresh intact sperm cells, the O_2 consumption of which is not markedly enhanced by the addition of succinate. The difference in enzymic behaviour between the intact and disrupted sperm cells has something of a parallel in the activity of blood carbonic anhydrase which can be demonstrated much more readily in laked than in unlaked, erythrocytes (Keilin and Mann, 1941).

The heads of ram spermatozoa separated by the process of mechanical disintegration can be further freed from lipoprotein and from adhering particles of middle-pieces and tails by repeated washing and differential centrifugation. Preparations obtained in this way consist of sperm-heads only; they were found to contain 3·9–4·3% phosphorus, all of it accounted for by deoxyribonucleic acid, but were free from lipid and acid-soluble phosphorus compounds.

Protein-bound iron, zinc and copper

Zittle and Zitin (1942*b*) found that the total iron content of dried lipid-free bovine epididymal sperm is about 7 mg./100 g., more iron being present in the middle-pieces and tails than in the heads. Of the total iron, 60% was extractable with pyrophosphate and trichloroacetic acid at 100°, and was therefore assumed to be of non-haematin nature; an attempt to identify haematin in the non-extractable portion was unsuccessful. However, with the aid of a spectroscopic method (Mann, 1937, 1938) designed specifically for

TABLE 12. *Distribution of total iron, zinc and copper in ram semen* (Mann, 1945*a*)

	100 ml. semen contain	
	In sperm (23 ml.; $3{\cdot}5 \times 10^{11}$ cells)	In seminal plasma (77 ml.)
Fe (mg.)	0·68	0·16
Zn (mg.)	0·70	0·28
Cu (mg.)	0·12	0·05

determination of haematin (as pyridine haemochromogen) in animal and plant tissues, the author was able to detect readily and to determine quantitatively haematin in bull as well as in ram spermatozoa. The distribution in ram semen, of total iron, and also of zinc and copper, is shown in Table 12 (Mann, 1945*a*). It can be seen that in the ram the concentration of these three elements is much higher in the spermatozoa than in the seminal plasma. With the aid of a Mickle disintegrator, it was possible to separate the heads from the tails and middle-pieces and to obtain sperm-head preparations which contained some iron but were completely free from haematin. On the other hand, the 'homogenates' from disintegrated tails and

middle-pieces contained a high proportion of iron in the form of haematin, as can be seen from Table 13. Iron as well as copper and zinc, present in the tail and middle-piece of ram spermatozoon, is largely non-dialysable. Iron occurs mainly as haematin some of which appears to be free and the rest protein-bound, mostly in the form of cytochrome. Copper belongs to a protein complex which readily gives up the metal on treatment with acid, thus resembling haemocuprein, the copper-protein isolated some time ago from blood cells (Mann and Keilin, 1938). Zinc also forms a complex with a protein but unlike the zinc-protein of blood cells it has negligible carbonic anhydrase activity.

TABLE 13. *Content of total iron, haematin, zinc, and copper, in mechanically disintegrated middle-pieces and tails of ram spermatozoa*

(Spermatozoa separated from seminal plasma by centrifugation and washing; mechanically disrupted in Mickle's disintegrator; sperm-heads removed by centrifugation. The supernatant fluid ('homogenate') which represents disintegrated middle-pieces and tails was analysed before and after dialysis. Its dry weight, expressed as mg./100 ml. semen, was 3300 before, and 2960 after, dialysis.)

	In the homogenate from mid-pieces and tails			
	Total contents		Non-dialysable	
	(mg./100 ml. semen)	(mg./100 g. dr. wt. of homogenate)	(mg./100 ml. semen)	(mg./100 g. dr. wt. of homogenate)
Total Fe	0·79	23	0·78	26
Haematin Fe	0·62	19	0·60	20
Zn	0·82	24	0·81	27
Cu	0·09	3	0·09	3

Human semen, in contrast to ram, has a much higher zinc content. The first to take notice of this were Bertrand and Vladesco (1921) who found 5·3–22·0 mg. Zn/100 ml. semen. More recently, the problem of seminal zinc was taken up by Mawson and Fischer (1953) who found that apart from the high zinc content of human seminal plasma which is derived from the prostatic secretion (see p. 19), centrifuged spermatozoa of man also carry a considerable zinc reserve of their own, nearly 2 mg./g. dry matter. Of this, however, only a minute fraction is endowed with carbonic anhydrase activity.

Sea-urchin semen has approximately the same concentration of copper as ram semen, with a similar distribution of the metal between sperm and seminal plasma (Barnes and Rothschild, 1950).

Cytochrome

Early investigators of semen were well aware of the fact that spermatozoa give a positive indophenol reaction with the 'Nadi' reagent, particularly marked in the regions of the acrosome and middle-piece (Herwerden, 1913). Ostwald (1907), Voss (1922) and Sereni (1929), among others, made important contributions to the subject of sperm indophenol oxidase and noted that the intensity of the reaction increased towards the final stages of sperm maturation and after ejaculation.

When in 1925 Keilin discovered cytochrome and identified indophenol oxidase with cytochrome oxidase, he noticed that of all the organs of a perfused frog, the heart muscle and the testicular tissue exhibited the strongest absorption spectrum of cytochrome. A little later, Brachet (1934) reported the presence of cytochrome in frog sperm, and Ball and Meyerhof (1940) in sea-urchin spermatozoa. In spite of that, attempts by several workers to detect the spectrum of cytochrome in mammalian spermatozoa met with failure and the functioning of cytochrome in mammalian semen continued to be deduced only indirectly from the evidence based on the oxidation of succinate and phenylenediamine (Shergin, 1940; Lardy and Phillips, 1941*c*; Zittle and Zitin, 1942*a*; MacLeod, 1943*a*). This led to some speculation, particularly in the case of human semen, about the mechanism of respiration, the more so, since it has been asserted that the oxygen consumption of human semen is associated predominantly with the seminal plasma and not with the spermatozoa themselves (MacLeod, 1941*a*; Ross, Miller and Kurzrok, 1941; Zeller, 1941).

In an effort to re-examine the whole problem, the author made a study of the cytochrome content of mammalian spermatozoa (Mann, 1945*a*, *c*). With the aid of the microspectroscope, an instrument eminently suitable for direct observation of absorption bands in tissues, no difficulty was experienced in the detection of the complete cytochrome spectrum in both ram and bull semen. Human semen has a sperm density at least ten times lower than bull semen,

but even here, the demonstration of the absorption bands of cytochrome became possible (Mann, 1951*a*) by the application of the technique of Keilin and Hartree (1949, 1950), whereby manifold intensification of absorption bands is brought about by means of liquid air.

Our evidence for the occurrence and active participation of the cytochrome system in the oxidative metabolism of mammalian spermatozoa can be briefly summarized as follows. Whole fresh semen examined a little while after ejaculation shows the diffuse spectrum of oxidized cytochrome as well as, weakly, the absorption bands of reduced cytochrome *a*, *b*, and *c*. However, after the addition of a reducing agent or on anaerobic incubation of the semen, the bands of the reduced cytochromes become much more pronounced, the cytochrome *a* band being more distinct than *c*, and the latter stronger than cytochrome *b*. The picture is similar with washed sperm suspensions in fructose-Ringer-phosphate solution; freshly prepared sperm suspensions show mainly the spectrum of oxidized cytochrome, which becomes reduced in the course of anaerobic incubation; on aeration of the incubated suspension cytochrome reverts to the oxidized form. The band of cytochrome *a* can be shown to undergo a typical change under the influence of carbon monoxide; the reaction product thus formed in the sperm resembling closely the carbon monoxide compound of cytochrome oxidase or cytochrome a_3, originally described by Keilin and Hartree (1939) in heart muscle preparations. Carbon monoxide, cyanide, azide, hydroxylamine, and other typical inhibitors of the cytochrome system in respiring tissues, all inhibit also sperm respiration.

An elegant experiment on the behaviour of cytochrome in spermatozoa was performed by Rothschild (1948*a*, *d*) who demonstrated that the oxygen uptake of sea-urchin sperm is inhibited by carbon monoxide and that the inhibition can be completely reversed by white light but not by light of the 548 mμ wavelength; the non-reversal at that particular wavelength being due to lack of absorption by cytochrome oxidase in this region of the spectrum. By interposing between the source of light and the microscope a colour filter transmitting light of the 548 mμ wavelength, Rothschild was able to observe spermatozoa microscopically, in the presence of

carbon monoxide, as if they were in the dark. Under these conditions, he found that carbon monoxide depressed the respiration without a corresponding decrease in sperm motility. A similar conclusion that respiration can be dissociated from motility was reached by Robbie (1948) from his study of the effect of cyanide on the spermatozoa of the sand-dollar (*Echinarachnius parma*).

The cytochrome system of sea-urchin spermatozoa includes cytochrome *e* and in this respect, it differs from mammalian sperm (Keilin and Hartree, 1949). Starfish (*Asterias forbesii*) spermatozoa on the other hand, exhibit a spectroscopic pattern of cytochrome very similar to sea-urchins (Borei and Metz, 1951). There is also some evidence that cytochrome occurs in plant sperm. This follows from the observation by Rothschild (1951*c*) that the movements of bracken spermatozoids (*Pteridium aquilinum* (L.) Kuhn) are photoreversibly inhibited by carbon monoxide.

The information gained by Zittle and Zitin (1942*a*) from experiments on the oxidation of *p*-phenylenediamine by spermatozoa disintegrated by sonic treatment, coupled with earlier observations on the indophenol colour reaction, indicated that the cytochrome system is located in the cytoplasm of the middle-piece and tail, rather than in the sperm-head. Our own spectroscopic studies led us to the same conclusion; the examination of the disintegrated middle-pieces and tails revealed the presence of all three cytochromes with cytochrome *a* predominating; these 'homogenates' oxidized rapidly both *p*-phenylenediamine and succinic acid, and the rate of oxygen consumption could be substantially increased by the addition of cytochrome *c*.

Catalase

It did not escape Miescher's notice that salmon spermatozoa differ from other cells by their restricted ability to decompose hydrogen peroxide. Some slight catalase activity has been reported in mammalian semen (Shergin, 1940) but it is questionable whether this was due to the spermatozoa themselves or to some accidental contamination of semen with blood, pus or bacteria. The deficiency of catalase in normal and cleanly collected bull semen is in fact, so typical that Blom and Christensen (1944, 1947) base on it a method for rating the 'hygienic quality' of bull semen; the test is carried out in Denmark

in special 'catalase tubes' in which hydrogen peroxide is added to semen and the volume of evolved oxygen recorded. In ram spermatozoa, even after mechanical disintegration, we were able to detect only a very weak catalase activity: an extract from 0·2 g. sperm (wet weight) required 20 min. at 18° to decompose a quantity of hydrogen peroxide which would have been decomposed in 2 min. by 0·001 ml. blood. Sea-urchin semen on the other hand, contains much more catalase (Evans, 1947; Rothschild, 1948*c*, 1950*c*; Barron, Gasvoda and Flood, 1949; Rybak and Gustafson, 1952).

The lack of catalase in mammalian semen explains the harmful effects of hydrogen peroxide and pure oxygen on spermatozoa (see p. 58). It is also of considerable physiological interest for another reason, inasmuch as the spermatozoa themselves produce hydrogen peroxide *in vitro* during the oxidation of certain amino-acids (see p. 117).

Hyaluronidase and other 'lytic' agents

The term 'hyaluronidase' in its widest sense, designates the mucolytic enzyme, or rather a group of enzymes, which bring about the depolymerization and hydrolysis of hyaluronic acid. The mucopolysaccharide called hyaluronic acid is a polymer of the disaccharide hyalobiuronic acid which consists of *N*-acetylglucosamine and D-glucuronic acid; its enzymic degradation, that is depolymerization and hydrolysis, is believed by Meyer and his school (1937, 1952) to be due to the opening of the *N*-acetylglucosaminidic bonds. Thus it should be possible to assess the activity of hyaluronidase by the determination of the reducing groups liberated by the enzymic process. In actual practice, however, this is only possible with the use of purified hyaluronidase since crude enzyme preparations often liberate additional reducing groups through the formation of free glucuronic acid and *N*-acetylglucosamine by β-glucuronidase and β-glucosaminidase, respectively. Apart from the 'reductimetric' method, however, there are several other ways in which the activity of hyaluronidase can be measured; among those in use is the 'mucin clot prevention (m.c.p.) test' in which the precipitation by acetic acid of the clot-like protein-hyaluronic acid complex is prevented by the enzyme; the 'turbidimetric' method is based on the observation that purified hyaluronate at pH 4·2, gives

a fairly stable colloidal suspension with dilute serum, whereas depolymerized hyaluronate under identical conditions remains clear; the 'viscosity reduction (v.r.)' method measures the decline in viscosity caused by depolymerization; in the so-called 'Spinnbarkeit'-method the stringiness of hyaluronic acid is assessed by means of a special device, before and after enzymic treatment. Each of the above methods, however, is open to criticism and limited in its scope (cf. Lundquist, 1949*a*; Swyer and Emmens, 1947; Meyer and Rapport, 1952).

The mammalian testis and sperm are the richest animal sources of hyaluronidase. The existence in testes and spermatozoa of a 'spreading' or 'diffusing' factor which, when injected intradermally, increases the permeability of the skin to fluids, was established by Hoffman and Duran-Reynals (1931) and McClean (1930, 1931); but Chain and Duthie (1939, 1940) deserve the credit for being the first to show that purified preparations of the testicular spreading factor possess strong hyaluronidase activity. Their finding was soon confirmed by other workers who made several attempts to purify the enzyme (Hahn, 1943; Freeman, Anderson, Webster and Dorfman, 1950; Tint and Bogash, 1950). The best preparations of bovine testicular hyaluronidase so far available are over ten thousand times more active than the testicular tissue itself, but as yet, even the most highly purified enzyme does not appear to be a homogeneous protein.

Hyaluronidase originates in the seminiferous epithelium of the mature testis, and in semen it is associated with the spermatozoa and not with the seminal plasma (Werthessen, Berman, Greenburg and Gargill, 1945; Joël and Eichenberger, 1945; Kurzrok, Leonard and Conrad, 1946; Swyer, 1947*a*; Jacquet, Plessis and Cassou, 1951). The content of hyaluronidase per sperm cell is highest in rabbit and bull; there is less of it in human and boar sperm, very little in dog, and practically none in birds and reptiles.

Although it is actually a part of the sperm cell, hyaluronidase is nevertheless so readily released by spermatozoa into the surrounding medium that it must be assumed to be located somewhere very close to the cell surface, possibly on the sperm-head (Hechter and Hadidian, 1947; Johnston and Mixner, 1947; Perlman, Leonard and Kurzrok, 1948). A few hours' freezing of an aqueous sperm suspension at $-10°$, or 24 hours' standing at 0°, has been found by

Swyer (1947*a*, *b*) to be quite sufficient for hyaluronidase to pass completely into solution. In an isotonic medium spermatozoa also tend to liberate hyaluronidase but when the concentration of the enzyme outside the cell reaches a certain level it seems to prevent further leakage. Indeed, under certain experimental conditions, spermatozoa depleted of hyaluronidase have been found to be capable of reabsorbing the enzyme from a hyaluronidase-rich medium (Emmens and Swyer, 1948; Swyer, 1951).

The physiological role of sperm hyaluronidase is far from clear at present. It may be related in some as yet unknown manner, to the spermiogenic function of the testis, but there is also some indication that in certain mammals at any rate, hyaluronidase acts by facilitating the contact between the male and female gametes through a direct liquefying action on the viscous gel which cements the follicle cells around freshly ovulated eggs.

Much thought has been devoted in the past to the problem of the participation of 'lytic' agents in the process of ovum fertilization, and to what at one time used to be called the 'ovulase' activity of spermatozoa. However, the early investigators of this problem were in the main concerned with lower animals. In many molluscs, fishes and amphibia, the unfertilized egg is normally surrounded by a viscous 'jelly coat' and a membrane, which the spermatozoon must penetrate before fertilization can be effected. To explain this process, several investigators postulated the presence of lytic agents in the spermatozoa, capable of mediating the fusion of the gametes, but there has been little evidence that these agents are in fact enzymic in nature, until Tyler's (1939, 1942) discovery of the 'egg-membrane lysin', a heat-labile protein-enzyme which he extracted from the sperm of two molluscs, the key-hole limpet *Megathura crenulata* and the abalone *Haliotis cracherodii*. With sperm extracts of these species, the disappearance of the egg membrane could be demonstrated within about 3 min., if the gelatinous coat of the egg was present, and in less than 30 sec., if the coat has been first removed.

The occurrence of similar lytic enzymes in the sperm of other lower animals is still under discussion (cf. Tyler, 1948; Berg, 1950; Runnström, 1951). Several lytic agents have been described in sea-urchin spermatozoa. One of them is the previously mentioned

androgamone III ('egg-surface liquefying agent', 'sperm lysin') discovered by Runnström and his co-workers and shown to be a heat-stable alcohol-soluble substance, probably a fatty acid (p. 71). The other is the protein-like jelly-coat 'dissolving' or 'precipitating' factor, identical with Hartmann's androgamone II; the disappearance of the egg-jelly under the influence of this protein-factor was originally described by Hartmann and his colleagues in *Arbacia pustulosa*, but a similar phenomenon was later observed in other sea-urchin species as well (Tyler and O'Melveny, 1941; Monroy and Ruffo, 1947; Kraus, 1950; Vasseur, 1951; Monroy and Tosi, 1952). The suggestion has been put forward that the agent which helps the sea-urchin sperm to penetrate the jelly-coat, is a mucopolysaccharase similar even though not identical, with hyaluronidase. This hypothesis, however, is in want of experimental support. It is also difficult as yet, to assign any definite role in fertilization to the proteolytic gelatin-liquefying enzyme which Lundblad (1950) extracted from the sperm of *Arbacia lixula* and *Paracentrotus lividus*.

In mammals, the existence of an enzymic 'cumulus-dispersing factor' was first brought to light by Yamane (1935), Pincus (1935) and Pincus and Enzmann (1936) who showed that both sperm suspensions and extracts from rabbit spermatozoa, brought in contact with unfertilized rabbit ova, can disperse within a short time the follicle cells of the cumulus oöphorus. In 1942, McClean and Rowlands discovered that hyaluronidase which they obtained not only from testes or spermatozoa, but also from snake venom and bacteria, can act as a cumulus-dispersing factor by liquefying the viscous gel which cements the follicle cells around freshly ovulated rat ova. Similar results on the mouse were reported by Fekete and Duran-Reynals (1943) who also noted that the gel of the cumulus responds to metachromatic staining with toluidine blue like hyaluronic acid.

It remains one of the unsolved mysteries in the phenomenon of fertilization that although the actual fertilization consists ultimately of the fusion of a single spermatozoon with the ovum, this can take place apparently only after a multitude of spermatozoa have reached the site of fertilization. Moreover, the denudation of the ovum from follicular cells has also been claimed to require the presence of numerous spermatozoa. According to McClean and Rowlands

(1942) they are needed to create and keep up a sufficiently high concentration of hyaluronidase to permit the denudation of the egg. This hypothesis was put to the test by Rowlands (1944) who found that it is possible to increase the fertilizing capacity of a subnormal number of rabbit spermatozoa by the addition of hyaluronidase in the form of extracts from whole dilute rabbit semen. Similar results were reported by Leonard and Kurzrok (1945, 1946).

In view of all this, little wonder that many investigators became attracted by the possibility of the therapeutic application of hyaluronidase in infertility. In fact, several enthusiastic reports appeared of success in human infertility of oligospermic origin, achieved by the addition of bovine testicular hyaluronidase to human semen. However, subsequent investigations failed to bear out the claim that hyaluronidase can enhance the fertilizing capacity of spermatozoa. According to Chang (1947*b*, 1949) the earlier positive results obtained with extracts from whole semen should be attributed not to hyaluronidase but to the effect of the seminal plasma as such. Similarly, the concept that a high sperm concentration in the vicinity of the egg is needed to denude the ovum from its cumulus, has been questioned by Leonard, Perlman and Kurzrok (1947), Austin (1948), and Austin and Smiles (1948) who demonstrated clearly that spermatozoa can in fact penetrate rat ova which are still enclosed in the cumulus. Presumably, the hyaluronidase charge carried by the spermatozoa makes it possible for the individual sperm to 'burrow' its way through the viscous gel which cements the follicular cells. This, however, need not necessarily involve the dispersion of the cumulus, which process is probably aided by the mechanical action of the cilia or by some other tubal factor.

Another development in the field of sperm hyaluronidase pertaining to the role of this enzyme in fertilization, has been the attempt to use certain inhibitors of hyaluronidase as systemic contraceptives. Among the inhibitors of hyaluronidase, presumably competitive in nature, are several derivatives of hyaluronic acid obtained by acetylation or nitration, also heparin, and a number of other anticoagulants and mucopolysaccharides, including a substance present in blood serum (Ferraro, Costa and Pelegrini, 1948; Hadidian and Pirie, 1948; Pincus, Pirie and Chang, 1948; Meyer and Rapport, 1952). Another two groups of inhibitors, some of

which act irreversibly, consist of heavy metals and quinones, including certain quinoid compounds derived from flavonoids (Beiler and Martin, 1947, 1948; Rodney *et al.*, 1950). Much interest was aroused some time ago by a report that phosphorylated hesperidine, a potent *in vitro* inhibitor of hyaluronidase, can act as an 'antifertility factor' when administered to mice and human beings; however, attempts to substantiate this claim have, so far, been unsuccessful (Martin and Beiler, 1952; Sieve, 1952; Martin, 1953; Chang and Pincus, 1953; Thompson, Sturtevant and Bird, 1953).

Sperm nucleoproteins

In the mature sperm cell, the sperm nucleus fills the head almost completely, the surrounding cytoplasm being very scanty. This nucleus consists of closely packed chromatin embedded in a relatively small amount of nuclear sap. The first to investigate the chemistry of the sperm nucleus was Miescher (1878, 1897) whose pioneer studies laid a foundation for the modern developments in the chemistry and physiology of the cell nucleus, the nucleoproteins and the nucleic acids. As a result of Miescher's brilliant researches, continued and extended by such investigators as Kossel, Schmiedeberg, Burian, Levene, Steudel, Lynch, Hammarsten, Rasmussen and Linderstrøm-Lang, and many others, it is now generally recognized that the chief component of sperm chromatin, one which confers upon the paternal (haploid) chromosomes their functions as transmitters of inheritance, is by its chemical nature, a *deoxyribonucleoprotein*, and consists of *deoxyribonucleic acid* conjugated with certain *basic nuclear proteins* such as *protamines* and *histones*.

Miescher used for his work chiefly salmon spermatozoa which he obtained mostly by stripping the live fish. A considerable advantage of this method is that the material thus obtained consists entirely of ripe spermatozoa and is therefore of uniform composition. This useful material, however, is not always procurable and is sometimes replaced by whole excised fish testes which are less suitable as they may contain some immature spermatozoa even during the breeding season. Miescher's routine procedure was to remove first the sperm-tails by plasmolysis with water or weak acetic acid and then to treat the washed suspensions of sperm-heads with ethanol and ether, to remove the lipids. In order to separate sperm nucleic

acid from the nuclear protein, Miescher treated the lipid-free material first with cold mineral acid (e.g. 0·25–0·5% hydrochloric acid) to remove the protein, and then with sodium hydroxide 'until the mixture tasted distinctly caustic to the tongue', to extract nucleic acid from the residue.

The deoxyribonucleoproteins in the spermatozoa of a great many fishes, including salmon, belong to the group of *nucleoprotamines* which can be extracted from the sperm nuclei with salt solutions. A convenient method for such an extraction and purification has been described by Pollister and Mirsky (1946). Spermatozoa of the brown trout, *Salmo fario*, were 'homogenized' with 1M solution of sodium chloride in a Waring mixer and the very viscous extract poured into six volumes of water; this caused the precipitation of the nucleoprotamine in the form of long strands, so fibrous that they could be wound around a glass rod and transferred in this way to another container. The fibrous material was dissolved in M-NaCl and reprecipitated with water, and then washed successively with 65% ethanol, hot 95% ethanol, and finally with ether; at this stage it contained about 6% phosphorus and 18% nitrogen, and consisted of deoxyribonucleic acid and protamine in a 6 : 4 ratio. When this material was dissolved in M-NaCl and dialysed against M-NaCl, the protamine slowly passed through the cellophane membrane leaving behind the solution of deoxyribonucleic acid. On pouring this solution into five volumes of ethanol, a fibrous precipitate of the nucleic acid was obtained, which, after drying, had a content of 8·97% phosphorus and 14·47% nitrogen. This composition approaches the theoretical value for the sodium salt of deoxyribonucleic acid, 9·28% phosphorus and 15·58% nitrogen.

The removal of protamine from nucleoprotamine solutions induces no significant change in viscosity. The high viscosity of nucleoprotamine solutions is due entirely to the highly polymerized nucleic acid. Deoxyribonucleic acid prepared, for instance, from herring spermatozoa, has a molecular weight of 800,000; each molecule of it requires some 100 molecules of protamine to form a molecule of nucleoprotamine. According to some calculations by Felix (1952), a single fish sperm nucleus contains $4{\cdot}5 \times 10^6$ nucleoprotamine molecules, enough to provide about 190,000 molecules per each chromosome, or several thousand per each gene.

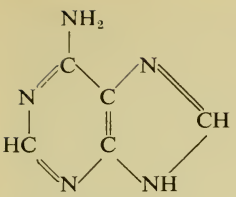
Unfortunately however, the extraction with M-NaCl is not a universal means for the separation of sperm nucleoproteins. In the key-hole limpet or freshwater clam, the sperm nucleoprotein resists extraction with M-NaCl, but can be brought into solution with a 2M salt solution, whereas no nucleoprotein can be extracted with NaCl of either concentration from the sperm of man, bull, boar or ram. Moreover, dialysis against M-NaCl or extraction with dilute mineral acids both prove inadequate for the removal of nuclear proteins from mammalian spermatozoa. In such cases, the separation of protein can be brought about with a chloroform-octanol mixture (Sevag, Lackman and Smolens, 1938), but before this is applied it is necessary to separate the sperm nucleus from the remainder of the sperm cell by ultrasonic or mechanical treatment.

Deoxyribonucleic acid

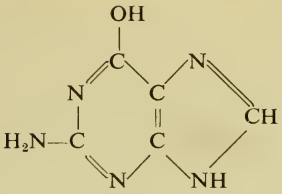
This when freed from nuclear protein, is composed of mono-nucleotides, each consisting of one molecule of phosphoric acid, one molecule of the sugar D(−)2-deoxyribose, and one molecule of a purine or pyrimidine base: adenine, guanine, cytosine or thymine. A small amount of yet another base, 5-methylcytosine (Wyatt, 1950, 1951), has been found so far in the sperm deoxyribonucleic acid of man, bull, ram, herring and sea-urchin (*Echinus esculentus*), but probably it occurs also in other species.

In all species, deoxyribonucleic acid is confined entirely to the sperm nucleus as can be demonstrated by various staining methods, and particularly by the '*Feulgen nucleal reaction*'. This reaction was described by Feulgen (1914, 1917) at first as a colour test for thymo-nucleic acid, but later it was adapted for the staining of cell nuclei (Feulgen and Rosenbeck, 1924).

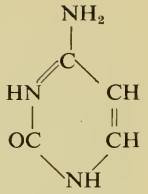
With thymonucleic acid itself, the test is carried out best on a solution of sodium nucleinate (0·1 g./1 ml.) prepared in a boiling tube on the water-bath. The solution is treated with 1 ml. 2N-H_2SO_4, and left at 100° for 3 minutes, then cooled and neutralized. When a drop of the hydrolysate is mixed with a few ml. of Schiff's fuchsin-sulphurous acid reagent (a 0·5% solution of fuchsin decolorized with SO_2 and the excess of SO_2 removed by suction), an intense purple colour develops. The chemistry of the Feulgen colour re-action is as yet only partly understood but is believed to involve the



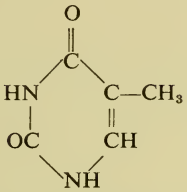
Adenine
(6-aminopurine)



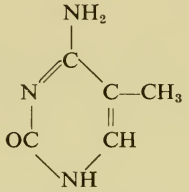
Guanine
(2-amino-6-hydroxy-purine)



Cytosine
(2-hydroxy-6-aminopyrimidine)



Thymine
(5-methyl-2 : 6-dihydroxy-pyrimidine)



5-Methyl-cytosine

following steps: acid hydrolysis which splits off purine and exposes deoxyribose; transformation of deoxyribose into ω-laevulinic aldehyde; lastly, formation of a purple-coloured reaction product of this labile aldehyde with fuchsin-sulphurous acid. The mechanism of the Feulgen reaction as exhibited by the cell nuclei in histological preparations, appears to be even more involved (Danielli, 1947; Davidson, 1950). In cellular material, however carefully carried out, the Feulgen reaction cannot be expected to yield results as accurate and specific as the colour test with pure deoxyribonucleic acid. Some of the difficulties inherent in the application of the reaction as a staining method for sperm nuclei, have been pointed out by Feulgen and Rosenbeck themselves, who noted that when fresh smears of human semen were treated with Schiff's fuchsin-sulphurous acid reagent, the sperm-heads stained rather weakly, whereas the middle-pieces and tails, though devoid of nuclear material, stained strongly. This observation was followed by a demonstration that the fuchsin-staining material present in the middle-piece and tail is a lipid, 'plasmal', which can be distinguished from the

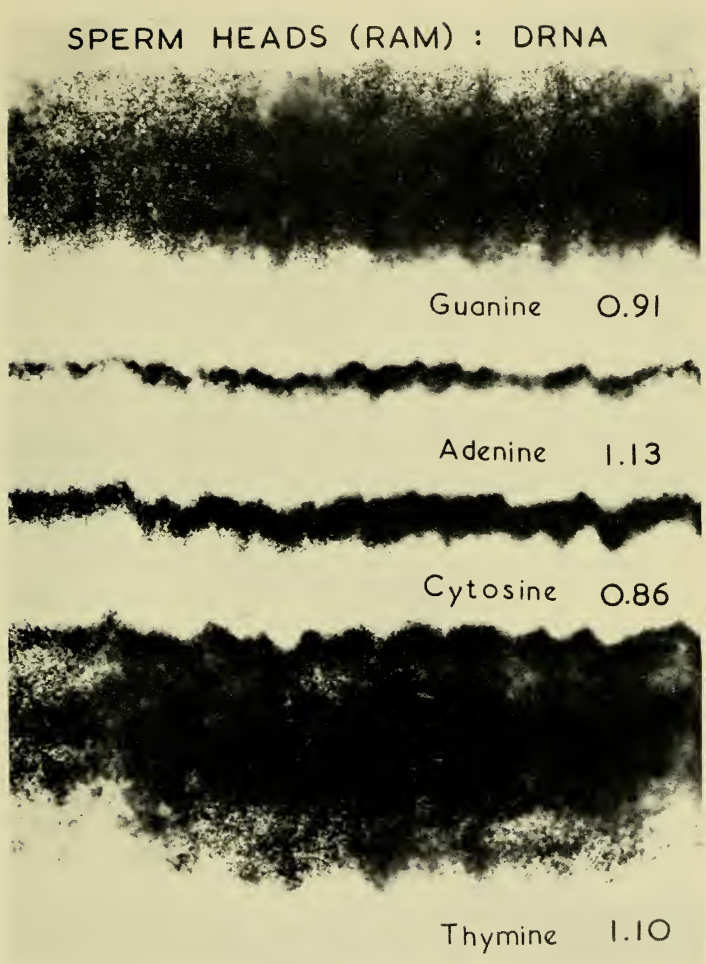
nucleoprotein of the sperm-head by its solubility in ethanol. Similar observations with animal sperm were later reported by other investigators. There is no doubt, however, that if carried out properly and under conditions which eliminate interference from other fuchsin-staining substances, the Feulgen nucleal reaction can be made specific for the sperm nucleus. Several authors have stressed the fact that the base and the posterior region of the sperm-head stains particularly strongly (Marza, 1930; Wislocki, 1950; Friedlaender and Fraser, 1952).

Although present in the sperm nuclei of all species, the composition of deoxyribonucleic acid varies somewhat from one species to another, with regard to the proportion of the various purine and pyrimidine bases. But within any given species, all body cells, including the spermatozoa, seem to yield on purification the same nucleic acid, that is one with the same molar ratio of adenine, guanine, cytosine and thymine. In the species so far examined, the ratio of adenine to thymine, of guanine to cytosine, and of total purines to total pyrimidines, was shown to equal unity.

The results of the analysis of bases in deoxyribonucleic acid from ram spermatozoa are shown in Plate V. The spermatozoa were disintegrated mechanically and suspensions of washed, tail-free sperm-heads were prepared (Mann, 1951*b*). Nucleic acid was separated from the nuclear proteins of the sperm-heads by the chloroform-octanol treatment, hydrolysed with formic acid, and the liberated bases separated on paper chromatograms by Wyatt's method, using as solvent an aqueous solution containing 65% *iso*propanol and 2N-HCl. The bases set free by acid hydrolysis and determined spectrophotometrically according to Markham and Smith (1949) were: guanine, adenine, cytosine and thymine in a molar ratio of 0·91 : 1·13 : 0·86 : 1·10. In addition, there was a small amount of methylcytosine which in Plate V is only just visible as a faint ultraviolet-absorbing band below cytosine; the molar ratio of cytosine to methylcytosine was 1 : 0·05.

Deoxyribonucleic acid (Na-salt) of human sperm was studied by Chargaff and his colleagues (Chargaff, Zamenhof and Green, 1950; Chargaff, 1951*a*, *b*); it contains 16% nitrogen and 8·9% phosphorus (Zamenhof, Shettles and Chargaff, 1950); the specific viscosity of a 0·135% solution in water equals 7·0, and the sedimentation constant

PLATE V



PURINE AND PYRIMIDINE BASES IN SPERM DEOXYRIBONUCLEIC ACID

Contact print, taken with ultraviolet light, of a paper chromatogram from the acid hydrolysate of ram sperm-heads. The figures indicate the molar ratios.

(S_{20}) for a 0·22% solution in 0·2M-NaCl is 5·7, a value which resembles closely that given by an undegraded specimen of calf thymus nucleic acid. According to Elmes, Smith and White (1952), the molar proportions of the purine and pyrmidine bases in deoxyribonucleic acid from human sperm and tissues are: guanine 0·92 (standard error of observations 0·036), adenine 1·23 (0·068), cytosine 0·84 (0·077), thymine 1·01 (0·09) and 5-methylcytosine 0·03.

TABLE 14. *Salmon sperm deoxyribonucleic acid* (Chargaff *et al.*, 1951)

(Molar ratios between the bases.)

Purines to pyrimidines	1·02
Adenine to thymine	1·02
Guanine to cytosine	1·02
Adenine to guanine	1·43
Thymine to cytosine	1·43

The analysis of the nucleic acid obtained from the sperm nucleoprotamine of salmon, *Salmo salar* (Chargaff, Lipschitz, Green and Hodes, 1951) gives a good illustration of the regularity in the composition of deoxyribonucleic acid. The nucleoprotamine was prepared by extraction with a salt solution, the nucleic acid set free by chloroform-octanol treatment, and after some further purification, recovered as the sodium salt. Two specimens were isolated in this way, containing 14·3% nitrogen and 8·9% phosphorus and 14·8% nitrogen and 8·9% phosphorus, respectively. The ultraviolet absorption spectrum measured in M-phosphate buffer pH 7·1, exhibited a maximum at 260 mμ. The specific viscosity in distilled water at 30·3°, was found to be 29·6 for a 0·22% solution, and 5·6 and 2·0 for 0·11 and 0·055% solutions, respectively. The bases were set free by acid hydrolysis and analysed chromatographically and spectroscopically. The ratios of the purine to the pyrimidine bases, of adenine to thymine, and of guanine to cytosine equalled unity; adenine and thymine exceeded guanine and cytosine by about 40% (Table 14).

Not only the composition but also the content of deoxyribonucleic acid in the cell nucleus appears to be fairly constant and characteristic for each animal species. The haploid nucleus of the sperm cell, however, differs from the diploid nuclei of the somatic cells in that it contains a reduced amount of chromatin and consequently it is

assumed to possess only one-half of the amount of deoxyribonucleic acid present in the somatic nuclei of a given species (Boivin, Vendrely and Vendrely, 1948; Vendrely and Vendrely, 1948, 1949, 1952; Mirsky and Ris, 1949, 1951; a review by Colette Vendrely, 1952). To illustrate this point, Table 15 gives data on the content of deoxyribonucleic acid in the sperm (haploid) nuclei and in the somatic (diploid or polyploid) cell nuclei of various species. The values range from $0{\cdot}11\times10^{-9}$ mg. for the diploid nucleus of a sponge cell to 168×10^{-9} for a diploid cell nucleus of Amphiuma, a urodele; the nucleic acid in the spermatozoa of the carp, trout, pike, tench, toad, cock and bull, is seen to be approximately one-half the content of the somatic cell nuclei. A high proportion of the data listed in Table 15, were obtained by means of the analytical procedures

TABLE 15. *Deoxyribonucleic acid content of single nuclei in somatic cells and spermatozoa*

(Contents expressed in mg.$\times10^{-9}$ deoxyribonucleic acid per cell nucleus; figures in brackets refer to authors.)

Animal	Type of cell	mg.$\times10^{-9}$/nucleus
SPONGES		
Orange sponge, *Dysidea crawshagi*	Diploid	0·11 (3)
COELENTERATE		
Jelly fish, *Cassiopeia*	Sperm	0·33 (3)
ECHINODERMS		
Sea-urchin, *Arbacia*	Sperm	0·67 (5)
Paracentrotus	Sperm	0·70 (5)
Echinometra	Sperm	0·98 (3)
Lytechinus	Sperm	0·90 (3)
Sea-cucumber, *Stichopus diabole*	Sperm	0·90 (3)
MOLLUSCS		
Limpet, *Fissurella barbadensis*	Sperm	0·50 (3)
Snail, *Tectarius muricatus*	Sperm	0·67 (3)
CRUSTACEANS		
Cliff crab, *Plagusia depressa*	Sperm	1·49 (3)
Goose barnacle	Sperm	1·46 (3)
FISHES		
Sturgeon, *Acipenser sturio*	Erythrocyte	3·2 (3)
Carp, *Cyprinus carpio*	Erythrocyte	3·2 (6)
	Sperm	1·6 (8)

Trout, *Salmo irideus* Gibb.	Erythrocyte	4·9 (6)
	Sperm	2·45 (6)
Salmo fario	Erythrocyte	5·79 (2)
	Sperm	2·67 (2)
Pike, *Esox lucius*	Erythrocyte	1·70 (6)
	Sperm	0·85 (6)
Tench, *Tinca tinca*	Erythrocyte	1·70 (6)
	Sperm	0·85 (6)
AMPHIBIANS		
Amphiuma	Erythrocyte	168 (3)
Frog	Erythrocyte	15 (3)
Toad	Erythrocyte	7·33 (2)
	Sperm	3·70 (2)
REPTILES		
Green turtle	Erythrocyte	4·92 (3)
BIRDS		
Domestic fowl	Erythrocyte	2·34 (2)
	Liver	2·56 (1) 2·39 (2)
	Spleen	2·54 (1)
	Kidney	2·20 (1)
	Heart	2·45 (1)
	Cock sperm	1·26 (2)
Duck	Erythrocyte	2·3 (7)
	Liver	2·1 (7)
MAMMALS		
Cattle	Liver	6·4 (4) 6·2 (2) 8·4 (3)
	Kidney	5·9 (4)
	Pancreas	6·9 (4)
	Thymus	6·4 (4)
	Bull sperm	3·3 (4) 2·82 (2)
Pig	Liver	5·0 (7)
	Kidney	5·2 (7)
Sheep	Liver	6·1 (7) 5·4 (7)
Dog	Liver	5·0 (7)
	Spleen	5·3 (7)
Man	Liver	5·9 (7)

(1) Davidson *et al.* (1950); (2) Mirsky and Ris (1949); (3) Mirsky and Ris (1951); (4) Vendrely and Vendrely (1948); (5) Vendrely and Vendrely (1949); (6) Vendrely and Vendrely (1952); (7) Vendrely (1952); (8) Vendrely and Vendrely (1953).

developed by Schmidt and Thannhauser (1945) and Schneider (1945, 1946) which are based largely on determinations of phosphorus and involve the removal of (i) the 'acid-soluble phosphorus compounds' (extraction with cold trichloroacetic acid), and (ii) the phospholipids (extraction with ethanol and ether), prior to the analysis of nucleic acid (see also Table 16).

The basic nuclear proteins, protamines and histones

The proteins conjugated with deoxyribonucleic acid are of the basic type and have been shown to be either protamines or histones in most instances so far examined. Protamines have been isolated from fish spermatozoa of several species. Of the various protamines, the best known are *salmine* from salmon and trout sperm, and *clupeine* from herring sperm. Much less is known about the other protamines, such as *scombrine* (mackerel), *cyclopterine* (lumpsucker), *esocine* (pike), *thynnine* (tunny fish), *percine* (perch), *cyprinine* (carp) and *sturine* (sturgeon).

Judging from the molecular weight and amino acid composition, salmine, clupeine and scombrine have a relatively simple structure, there being approximately two molecules of arginine to one molecule of monoamino acid. Thus, for example, the analysis of salmine sulphate prepared from the spermatozoa of the Spring or Chum salmon (Tristram, 1947, 1949) suggests a molecular weight of about 8000, with a total of 58 amino acid residues: 40 arginine, 8 isoleucine, 2 valine, 4 proline, 3 glycine, 1 alanine and 7 serine; this salmine sulphate contains 19·85% sulphuric acid, i.e. 40 equivalents per molecule, sufficient to combine with all arginine residues. Another salmine sulphate, one prepared from the sperm of the Columbia River salmon (Block and Bolling, 1945) is said to contain 67 amino acid residues: 47 arginine, 1 isoleucine, 3 valine, 6 proline, 4 alanine and 6 serine (see also: Corfield and Robson, 1953).

The results of formol titration indicate that salmine contains one free amino or imino group per molecule of 8000, and the end group assay carried out by means of the dinitrofluorobenzene method (Sanger, 1952) suggests that the *N*-terminal position is occupied by the imino group of proline. In clupeine, the *N*-terminal position is also taken up by proline, whereas at the other end of the amino acid

TABLE 16. *Distribution of phosphorus compounds in ram semen*

(Results based on analysis of 16·8 ml. ram semen, representing 14 ejaculates from 7 rams; average volume of single ejaculate 1·2 ml.; 3,050,000 spermatozoa/μl. Semen separated by centrifugation for 20 min. at 10,000 *g*, into sperm, 26% v/v, and seminal plasma, 74% v/v.)

	mg. P/100 ml. semen		
	In whole semen	In sperm	In seminal plasma
Total phosphorus	328·5	186·7	141·8
Acid-soluble phosphorus (in trichloroacetic acid extract)			
$P_{inorg.}$ (orthophosphate determined as $MgNH_4PO_4$)	10·3	3·1	7·2
P_0 (phosphate reacting directly with molybdate)	11·9	4·7	7·2
P_7 (phosphorus which appears as orthophosphate after 7 min. hydrolysis with N-HCl)	15·4	8·1	7·3
P_{30} (phosphorus which appears as orthophosphate after 30 min. hydrolysis with N-HCl)	15·9	8·6	7·3
$P_{tot.\ ac.\ sol.}$ (total acid-soluble phosphate determined after incineration)	159·4	27·4	132·0
P_{ATP} (labile phosphate of adenosine triphosphate determined in the Ba-salt by the method of Parnas and Lutwak-Mann, 1935)	4·3	4·3	0·0
P_{hexose} (phosphate of 6-phosphohexose determined in the supernatant from Ba-ATP)	6·2	6·0	0·2
Phospholipid phosphorus (extracted with ethanol and ether from the residue insoluble in trichloroacetic acid)	30·8	27·9	2·9
Deoxyribonucleic acid-phosphorus (extracted with KOH from the residue left after removal of acid-soluble P and phospholipid; precipitated from the KOH extract by acid)	111·0	111·0*	0·0
Residual phosphorus (left after removal of acid-soluble compounds, phospholipids and deoxyribonucleic acid)	27·3	20·4	6·9

* Corresponding to $3{\cdot}2\times10^{-9}$ mg. deoxyribonucleic acid/sperm cell.

chain, the *C*-terminal position is filled by arginyl-arginine (Dirr and Felix, 1932). Arginine-arginine linkages occur also with great frequency in the amino acid chain itself (Felix and Schuberth, 1942). The following peptides have been identified as breakdown products of a partial hydrolysis of clupeine: arginyl-arginine, triarginyl-arginine, alanyl-arginyl-arginine, seryl-arginyl-arginine, and alanyl-alanine; on the basis of the available evidence, Felix, Fischer and Krekels (1952) suggested the following sequence of proline (Prol.), arginine (Arg.) and monoamino acids (M.) in clupeine,

$$\text{Prol. (Arg. Arg. Arg. Arg. M. M.)}_n \text{ Arg. Arg.}$$

Salmine and clupeine, irrespective of their origin, both contain arginine as the sole basic amino acid; in the corresponding nucleoprotamines the ratio between the arginine residues of the protamines and the phosphoric acid equivalents of nucleic acid is not far from unity, usually about 0·95 (Felix, 1951). In other protamines, on the other hand, e.g. percine and sturine, a certain proportion of the basic units is present in the form of histidine and lysine.

On close inspection of analytical data relating to the various protamines, there stands out a considerable degree of variability in the amino acid composition, even in closely related species. The situation is even more complicated in sperm nucleoproteins which contain histones instead of protamines. The histones such as occur for instance, in the sperm nucleoproteins of sea-urchins (*arbacine* of Mathews, 1897) and cod-fishes (*gadushistone* and *lotahistone*) are characterized by a wider range of amino acids, including tyrosine. It is not improbable that some of the observed variations in the amino acid composition of protamines and histones, represent distinct species characteristics analogous to those which are encountered in other proteins, e.g. in the globins of various haemoglobins. On the other hand, however, the standards for the assessment of chemical purity of nuclear proteins are open to criticism, and it is uncertain whether the examined protamines and histones were always really pure. Quite likely a sperm nucleus may contain in some cases more than one basic protein, that is, a main protamine or histone, together with a smaller amount of a 'subsidiary' product (Stedman and Stedman, 1951). Furthermore, there is also the possibility that some of the reported differences in the composition of

nuclear proteins are simply due to the use of material containing variable proportions of mature and immature spermatozoa. Spermatocytic development is well known to be associated with characteristic changes in the amino acid composition of the nuclear proteins. Immature spermatozoa of salmon, for instance, obtained directly from excised testes, contain a histone instead of the salmine. Similarly, the testes of the mackerel yield on extraction with dilute hydrochloric acid a histone, 'scombron', instead of the protamine 'scombrine'. This and similar observations, prompted Kossel (1928) to acclaim the histones as 'intermediary stages' in the transformation of complex proteins into protamines.

The non-basic nuclear proteins; karyogen and chromosomin

In addition to the basic proteins, the sperm nucleus always contains some non-basic or so-called residual proteins. In lipid-free preparations from salmon sperm-heads, Miescher found 19·78% protamine (extracted with 0·25–0·5% HCl), 2·94% acid-soluble protein material other than protamine, 60·50% of nucleic acid (extracted with NaOH) and 16·78% of an iron-containing residue which was insoluble either in acid or in alkali and which he believed to contain 'karyogen', the 'inner-space protein substance' (*Innenraumsubstanz*), of the sperm nucleus.

It was found later that the residual or non-basic nuclear proteins, unlike the protamines and histones, contain tryptophan as a characteristic component. Opinions are divided, however, on the problem of the actual ratio of non-basic to basic proteins in the sperm nucleus. According to the Stedmans (1943, 1947), the nucleoprotamine present in the sperm-heads of salmon accounts for no more than 70% of the dry, lipid-free material, whereas the remainder is made up largely of a non-basic protein 'chromosomin', which contains tryptophan.* On the other hand, Pollister and Mirsky (1946) state that the nucleoprotamine present in trout spermatozoa

* The Stedmans' *chromosomin* must not be confused with *chromosin*, a name given by Mirsky and Pollister (1946) to a *complex* extracted with M-NaCl from isolated cell nuclei of various organs, including thymus, liver, spleen, pancreas, brain, frog testes and bacteria. This complex is composed of deoxyribonucleic acid, histone, and a tryptophan-containing protein.

accounts for as much as 91% of the dry, lipid-free mass of the head nuclei. These authors, however, also find in the nuclei a characteristic tryptophan-containing residual protein.

Keratin-like protein of the sperm membrane

Within the category of 'residual' sperm proteins are also certain highly insoluble sulphur-rich proteins, obtained from mammalian spermatozoa and derived probably from the sperm membrane. The first mention of a 'sulphur-rich substance, containing more than 4% S in the heads of bull spermatozoa', was made in 1878 by Miescher, who did not, however, investigate its origin and composition. Green (1940) extracted ram spermatozoa successively with dilute acid and alkali, and obtained a residue containing 19·3% nitrogen and 11·4% cystine, which he believed to represent the sperm membrane. The possibility of a keratin-like protein present in the sperm membrane is strengthened by the observations of Zittle and O'Dell (1941*a*) on the solubilizing action of thioglycolic acid and trimethylbenzylammonium hydroxide on bull spermatozoa. In boar sperm, the portion which remains undissolved after prolonged treatment with N-NaOH, consists of 'ghost' sperm-heads which resemble in shape the sperm membranes (Thomas and Mayer, 1949). A remarkable property of the sperm membrane which can be demonstrated microscopically, is the extraordinary elasticity of the sperm-head structure; this was convincingly demonstrated in the experiments of Moench and Holt (1929–32), who were able to hook the head of a human spermatozoon with a microsurgical needle and to stretch it very considerably.

CHAPTER V

Protein Constituents and Enzymes of the Seminal Plasma

Proteoses and free amino acids. Fibrinolysin and fibrinogenase. Pepsinogen. Ammonia formation. Amino acid oxidase. Seminal phosphatases; 'acid' and 'alkaline' phosphatase; 5-nucleotidase; pyrophosphatase. Enzymic hydrolysis of adenosine triphosphate.

Proteoses and free amino acids

A DISCUSSION of the nature of extracellular protein constituents of semen demands the recognition of certain circumstances which are peculiar to the seminal plasma. An important, though sadly neglected fact is that the protein content of the seminal plasma does not remain constant after ejaculation but undergoes rapid changes of enzymic character which manifest themselves in a progressive decrease in the concentration of non-dialysable protein-nitrogen and a simultaneous accumulation of non-protein nitrogen, free amino acids and, at a late stage, of free ammonia. Unless this is fully taken into account, results of protein and amino acid analysis in semen are but of little significance and yield no information on the initial distribution of nitrogenous compounds. This applies especially to human semen as was convincingly demonstrated by Lundquist (1949*c*, 1952), and also by Jacobsson (1950, Fig. 11).

Even in freshly collected seminal plasma a large proportion of total nitrogen is found partly as a protein-like material which passes readily through semi-permeable membranes but is not heat-coagulable and not precipitated by trichloroacetic acid; accordingly, this has been classified as propeptone, hemialbumose, primary proteose, and secondary proteose (thioalbumose and synalbumose) (Posner, 1888, 1892; Marshall, 1922; Goldblatt, 1935*a*). In human seminal plasma, out of a total content of about 3·5 to 5·5 g. protein-like material per 100 ml., no more than 18%, usually much less, is coagulated by heat, and about 60% passes through cellulose

membranes which are impermeable to blood serum proteins (Huggins, Scott and Heinen, 1942). The electrophoretic pattern of the non-dialysable portion from five different specimens of human seminal plasma has been examined by Gray and Huggins (1942) who observed four distinct components which corresponded to serum albumin (17·7–22·7%), α-globulin (19·8–27·8%), β-globulin (34·3–44·5%), and γ-globulin (11·4–21·0%). Ross, Moore and Miller

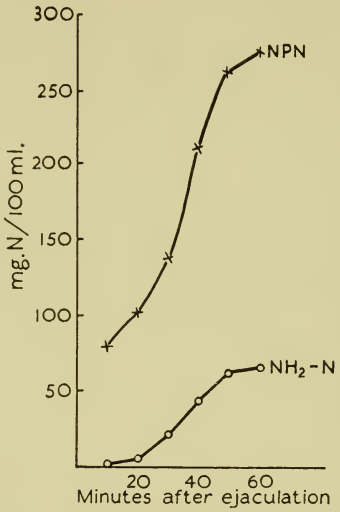


FIG. 11. *Increase of non-protein nitrogen and amino-nitrogen content in human semen on incubation at* 37°.

(Jacobsson, 1950)

(1942) also carried out an electrophoretic and chemical analysis of human seminal plasma in which they distinguished five protein fractions: albumin (less than 0·02%); 'nucleoprotein' (less than 0·04%); proteose ('P_1'), which was not heat-coagulable and passed through a membrane of 25 Å pore diameter; two water-insoluble proteins ('P_2' and 'P_3') and a mucoprotein ('P_4') which contained 9·3% N, 10·8% hexosamine, and gave on hydrolysis with N-HCl reducing substances (26·8%) but no uronic acid. The strongly positive periodic acid–Schiff reaction which is very characteristic

for human seminal plasma, is probably due to this mucoid substance (Wislocki, 1950).

The proteoses in trichloroacetic acid extracts from semen, which one encounters not only in man, but also in the ram, bull, boar, and other species, occasionally interfere with chemical analyses of certain non-protein constituents. To overcome this difficulty, it is advisable to replace trichloroacetic acid with other deproteinizing agents such as zinc hydroxide, tungstic acid, phosphotungstic acid or ethanol. Another source of trouble encountered in analytical work with semen and due to the mucinous substance in seminal plasma, human in particular, is that on centrifugation the mucus has a tendency to form a stringy mass which firmly adheres to the sperm cells. Caution must therefore be exercised in attributing to spermatozoa as such, analytical results obtained with centrifuged human semen.

Several assays of free amino acids in mammalian seminal plasma have been carried out, mostly however, by chromatographic or microbiological methods, and not by chemical isolation. A notable exception is the work of Wagner-Jauregg (1941) who isolated crystalline tyrosine from an ethanolic extract of human semen. The following free amino acids were found to occur in the seminal plasma of man, glycine, threonine, alanine, valine, leucine, isoleucine, cystine, proline, tyrosine, phenylalanine, lysine, arginine, aspartic acid and glutamic acid (Jacobsson, 1950; Lundquist, 1952). In bovine seminal plasma serine, glycine, alanine, aspartic acid and glutamic acid were found (Gassner and Hopwood, 1952), and a similar pattern was also observed in the seminal vesicle secretion and in the ampullar fluid, the latter containing in addition a trace of tyrosine. According to Gassner, the free amino acids in bull seminal plasma disappear after castration, like seminal fructose and citric acid, but their content is not restored by testosterone administration; furthermore, vasectomy alone, which is without effect on the content of fructose, causes a disappearance of amino acids from bull seminal plasma.

Free amino acids occur also in fish semen; as long ago as 1923, leucine, lysine and alanine have been isolated in pure form from protein-free extracts of herring testicles (Steudel and Suzuki, 1923).

There are indications that the amino acids and proteoses present

in the seminal plasma may be of some importance to the spermatozoa. It may be recalled that excessive dilution of semen exerts a deleterious effect on spermatozoa and that this can be counteracted, partly at least, by the inclusion in the diluting media of amino acids such as glycine, alanine, valine, leucine, lysine, and glutamic acid (p. 76). The beneficial action of these amino acids is believed to depend primarily on their metal-binding capacity (Tyler and Rothschild, 1951). Several other effects of amino acids have been observed with the sperm of lower animals. Giese and Wells (1952) found that glycine (0·05M) protected the spermatozoa of *Strongylocentrotus purpuratus* from the detrimental effect of light. Metz and Donovan (1950) demonstrated that in the starfish certain amino acids promote the agglutination of spermatozoa by egg-water of this species; in the absence of these amino acids agglutination does not take place.

Fibrinolysin and fibrinogenase

The seminal proteoses and amino acids are presumably the products of proteolytic activity which in the seminal plasma is derived mainly from the prostatic secretion, but partly also from the seminal vesicle fluid. The two powerful proteolytic agents of the prostatic secretion are 'fibrinolysin' and 'fibrinogenase' (see also pp. 17 and 29).

The coagulation of human semen is followed by liquefaction, a process which is catalysed by a proteolytic agent present in the prostatic secretion. Its discoverers, Huggins and Neal (1942) named it fibrinolysin because of its ability to digest blood fibrin, and its resemblance to the fibrinolytic agent in haemolytic streptococci (Tillet and Garner, 1933). However, the fibrinolytic system present in blood has now been resolved into several distinct components, whereas the streptococcal fibrinolysin has been defined as a kinase, i.e. an activator of the fibrinolytic enzyme preformed in the blood. Consequently, the name 'fibrinolysin' has been abandoned with reference to the streptococcal agent in favour of 'streptokinase'. Furthermore, it proved impossible to replace streptokinase by prostatic fibrinolysin as an activator of the blood enzyme (Oettlé, 1950).

The fibrinolytic activity can be assayed in human semen by the method of Harvey (1949), which consists in mixing a constant volume

of oxalated blood plasma with varying volumes of semen, inducing clotting by the addition of 1·5% calcium chloride, and noting the time required for the clot to liquefy. Owing to the inhibitory effect of blood plasma on fibrinolysis, the plasma must not constitute more than one-tenth of the reacting system. Harvey states that the degree of fibrinolytic activity in semen varies with the individual but she found no correlation between this activity and either the volume of ejaculates or any characteristics of spermatozoa. Similarly, there was no positive relationship between the lysin content and semen viscosity. However, specimens which were exceptionally viscous, usually also had low fibrinolytic power.

The precise nature of seminal fibrinolysin and its relation to plasmin, the fibrin-splitting agent of the blood, will not be known until the enzyme has been purified. Moreover, experiments by Kaulla and Shettles (1953) indicate that in addition to the plasmin-like enzyme proper, the human seminal plasma contains at least three other agents, (i) *fibrinolysokinase*, an activator of blood profibrinolysin, (ii) a small amount of *profibrinolysin* itself, i.e. of material which can be activated by streptokinase, and (iii) *antifibrinolysin*, an inhibitor of plasmin, which, however, is present in a much lower concentration in the seminal plasma than in blood serum.

Fibrinogenase is the name given by Huggins and Neal (1942) to the proteolytic agent, highly active in canine prostatic secretion, but less so in human prostatic fluid, which destroys blood plasma fibrinogen. Huggins and his co-workers (1942, 1943) also share the credit for having recognized the similarity between certain other proteolytic properties of the prostatic secretion and those of pancreatic trypsin. More recently, the 'tryptic' enzyme of human semen has been partially purified by Lundquist (1952), who defined as a unit the amount of enzyme which in 1 hr., at pH 7·6, and 37°, liberates from added casein a quantity of chromogen corresponding to 0·1 mg. free tyrosin; he achieved an activity of about 1 unit per mg. of protein-nitrogen, i.e. an approximately tenfold purification. The purified enzyme was active also towards haemoglobin and both human and bovine blood plasma fibrinogen. In an attempt to purify from human semen the natural substrate for the proteolytic activity, Lundquist obtained a protein fraction, 'seminal fibrin', which was readily digested by enzyme preparations both from

semen and the prostate gland, and yielded the same amino acids which appear normally in human semen.

Pepsinogen

Apart from the above mentioned protease which acts optimally at pH 7·6, two more proteolytic enzymes have been found in human semen by Lundquist and his co-workers (1951, 1952, 1953). One is an amino peptidase which hydrolyses leucine amide, glycyl-glycine, triglycine and glycyl-leucine, with an optimum around pH 7·5. The other has been identified as pepsinogen. According to Lundquist and Seedorff (1952), the activity of pepsinogen in semen corresponds to 2 μg. pepsin/ml., which is of the same order of magnitude as that found in gastric juice. But unlike the trypsin-like enzyme, seminal pepsinogen seems to originate in the seminal vesicles and not in the prostate. Its specific function is not fully understood as it is difficult to envisage in semen the high hydrogen ion concentration required for the conversion of pepsinogen to pepsin.

Ammonia formation

A phenomenon probably associated with the enzymic degradation of proteins, is the progressive accumulation of free ammonia which takes place in whole semen and in seminal plasma, on anaerobic as well as aerobic incubation. This has been observed in several species (Shergin, 1933; Mann, 1945*a*). In ram semen for example, the content of free ammonia (estimated by vacuum-steam distillation in the Parnas–Heller apparatus) was found to increase from 1·3 mg. NH_3-N/100 ml. in fresh semen to 9·7 mg. NH_3-N/100 ml., after 7 hr. incubation at 37°, under sterile conditions (Mann, 1945*a*). Ammonia formed in semen is in considerable excess of the amount which could be derived from adenyl derivatives; the total adenine amino-*N* content of the semen as assessed enzymically with heart muscle deaminase being only about 4 mg./100 ml. in fresh, and 3 mg./100 ml. in incubated, semen. It is also unlikely to originate from urea, since the content of urea in semen is not significantly affected by incubation.

Amino acid oxidase

In addition to sugars and fatty acids, spermatozoa are capable of oxidizing a number of amines and amino acids. The oxidative deamination of amino acids by bull spermatozoa has been the subject of a study by Tosic and Walton (1945; 1946*a*, *b*, 1950). The starting point of this study has been the observation that the addition of egg-yolk to bull sperm causes an increased oxygen uptake, which, however, gradually declines in about an hour's time. Egg-yolk fractionation led to a dialysable, nitrogen-containing fraction which was oxidized by spermatozoa. The oxidation was accompanied by accumulation of ammonia and formation of hydrogen peroxide. Evidence was obtained which pointed to peroxide being responsible for the gradual decline in the oxygen uptake by spermatozoa.

In the course of their study, Tosic and Walton examined several pure amino acids and found that spermatozoa oxidize three naturally occurring amino acids, namely L-tyrosine, L-phenylalanine and L-tryptophan. According to Tosic (1947, 1951), the hydrogen-peroxide-forming aerobic process in bull semen is an oxidative deamination catalysed by the L-amino acid oxidase of spermatozoa, which differs from the analogous enzyme of other animal tissues by having its range of activity restricted to only three aromatic amino acids; the activity of the enzyme can be expressed by the equation

$$\mathrm{R\,CH_2{\cdot}CH{\cdot}COOH + O_2 + H_2O \longrightarrow R\,CH_2{\cdot}C{\cdot}COOH + H_2O_2 + NH_3}$$

(the $\mathrm{NH_2}$ group is attached to the CH; the O is attached to the C)

Seminal phosphatases

Semen owes its powerful phosphatase activity mainly to the seminal plasma which carries several different dephosphorylating enzymes derived from the male accessory organs of reproduction. Among the most active and best known enzymes in this group are the so-called 'acid phosphatase' and 'alkaline phosphatase'. In addition to these two phosphomonoesterases, the seminal plasma contains '5-nucleotidase', a pyrophosphatase, and several adenosinetriphosphatases.

In early studies on phosphatases, the substrates commonly

used were α- and β-phosphoglycerol, and phosphohexoses, chiefly 6-phosphoglucose and 6-phosphofructose, but also 1 : 6-diphosphofructose. More recently, however, other organic phosphoric acid derivatives came into use, including various nucleotides and intermediary phosphorylated compounds of glycolysis, as well as two synthetic substances: phenylphosphate (King and Armstrong, 1934) and phenolphthaleine phosphate (King, 1943; Huggins and Talalay, 1945). The introduction of histochemical techniques marked another important development in studies on phosphatases (Gomori, 1939, 1941*a*, *b*, 1953). The histochemical investigations have thrown much light upon the pattern of phosphatase distribution in the male accessory organs and have helped to establish the existence of 'secretory' phosphatases, localized in the secretory epithelia and secretions of accessory glands, as distinct from the 'stromal' phosphatases which are present only in the stroma (Dempsey, 1948; Bern, 1949; Rollinson, 1954).

'Acid' and 'alkaline' phosphatase

An observation that the phosphatase activity of male urine is usually higher than in women, led Kutscher and Wolbergs (1935) to examine the phosphatase in semen and in the prostate gland. They soon found that semen and prostate are among the richest sources of acid phosphatase in the human body, the enzyme being optimally active at pH 5–6, equally well towards α- and β-phosphoglycerol, but largely inactive towards diphosphofructose and pyrophosphate (Kutscher and co-workers, 1936, 1938). Subsequent investigations confirmed and extended these findings; the demonstration by Scott and Huggins (1942) that, while the voided urine of man is rich in the enzyme, urine collected directly from the renal pelvis shows only little enzymic activity, was a convincing proof that the content of acid phosphatase in normally voided male urine is due largely to the admixture of prostatic secretion.

Acid phosphatase is an important secondary male sex characteristic. Investigations by Gutman and Gutman (1938*b*) have shown that the level of the enzyme in the human prostate is low in childhood but increases rapidly at puberty; thus the activity, expressed in King-Armstrong units per gram prostate tissue, was 1½ units at four years of age, 73 units at puberty, and 522 to 2284 units in adult men.

A similar relation to age was observed in monkeys and dogs; in both these species administration of androgenic hormones to immature males stimulates considerably the output of the enzyme from the prostate gland (Gutman and Gutman, 1939; Huggins and Russell, 1946). A certain correlation appears to exist in adult men between the level of acid phosphatase in semen and androgenic activity (Gutman and Gutman, 1940; Gutman, 1942; Engberg, Anderson, Sury and Raft, 1947). However, like other constituents of semen, the level of prostatic phosphatase activity varies from one species to another, as well as between individuals within the same species.

Under physiological conditions, acid phosphatase does not pass from the prostate into the blood stream. However, significant amounts of it appear in the blood plasma as a result of malignant growth in the prostate and metastases of prostatic cancer in the bones; injections of androgen still further increase the level of enzyme in blood plasma, whereas castration or treatment with oestrogens lead to a spectacular decrease. The determination of prostatic phosphatase activity in blood has been utilized as a valuable diagnostic aid in prostatic carcinoma and in the course of clinical treatment (Gutman and Gutman, 1938*a*; Huggins, Scott and Hodges, 1941; Watkinson, Delory, King and Haddow, 1944).

An important addition to our knowledge of the physiological functions of acid phosphatase in seminal plasma, has been the discovery made by Lundquist (1946) that freshly ejaculated human semen contains phosphorylcholine which on ejaculation is rapidly dephosphorylated by the acid phosphatase to free choline and orthophosphate. This phenomenon is more fully discussed elsewhere (p. 170). But there is evidence that apart from the acid phosphatase which acts on phosphorylcholine optimally at pH 6·3 (in acetate buffer), human seminal plasma contains another phosphatase which acts on the same substrate at a higher pH (Hudson and Butler, 1950). Of considerable interest is also the finding that the acid seminal phosphatase exhibits *in vitro* a distinct transferase activity (Green and Meyerhof, 1952); partially purified acid phosphatase from the human prostate has been shown to catalyse at pH 5·5 the transfer of phosphate from both β-phosphoglycerol and phosphocreatine to glucose; the product in each case was 6-phosphoglucose

(Morton, 1953). Methods for the purification of acid phosphatase from human prostate glands and semen have been described by London and Hudson (1953) and Boman (1954).

Alkaline phosphatase, like the 'acid' enzyme, is widely distributed in male accessory organs but its localization in cells and concentration in accessory gland secretions is different. Human semen with its conspicuously high level of acid phosphatase, has a low concentration of the alkaline phosphatase. Bull semen on the other hand, has only slight acid phosphatase activity but contains more of the alkaline phosphatase (Haq and Mullen, 1948; Reid, Ward and Salsbury, 1948*a*). This difference between the human and bovine semen is not altogether unexpected, since the bulk of bull seminal plasma is derived not from the prostate but from the seminal vesicles. In the rat both phosphatases are of low activity; with the exception of the ventral prostate which may contain up to 20 units of alkaline phosphatase per g. tissue, the level of either enzyme seldom exceeds 4 units per g. in any one of the other accessory organs. After castration the activity of both these enzymes diminishes first in the rat seminal vesicles, and a little later in the prostate; but the percentage decrease of enzymic activity and of organ weight is roughly equal (Huggins and Webster, 1948; Stafford, Rubinstein and Meyer, 1949).

TABLE 17. *Phosphatase activity of ram seminal plasma on phosphohexoses* (Mann and Lutwak-Mann, 1951*b*)

(The liberation of sugars was examined by incubating 5 mg. substrate (Na salt) with 1 ml. dialysed seminal plasma at pH 7 or 0·2 ml. dialysed seminal plasma at pH 9, for 1 hr., 37°, in the presence of 0·005M-$MgCl_2$. The sugars were determined after deproteinization with $ZnSO_4$ and $Ba(OH)_2$; glucose was estimated by means of glucose oxidase (Mann, 1944; 1946*b*).)

	Fructose (%)		Glucose (%)		Inorg. phosphate (%)	
	pH=7	pH=9	pH=7	pH=9	pH=7	pH=9
1-Phosphoglucose	1	1	65	92	67	93
6-Phosphoglucose	17	21	77	79	94	100
6-Phosphofructose	23	30	57	67	97	100
6-Phosphomannose	23	15	43	49	94	100
1 : 6-Diphosphofructose	7	70	5	15	20	95
1-Phosphofructose	60	96	0	4	60	100

Alkaline phosphatase has an optimum at about pH 9, and is capable of hydrolysing among others, 1-phosphofructose, 6-phosphofructose and 1 : 6-diphosphofructose. Our researches indicate that this activity may represent an essential step in the process of fructose formation and secretion by the accessory organs (Mann and Lutwak-Mann, 1951*a*, *b*) (see also p. 150). Ram seminal plasma is particularly rich in alkaline phosphatase which, though it acts optimally at pH 9, also shows appreciable activity towards phosphohexoses at pH 7. This can be seen from Table 17 which gives rates of dephosphorylation for various compounds. In the case of 6-phosphofructose and 1 : 6-diphosphofructose, some glucose is formed in addition to fructose, owing to the presence of phosphohexose isomerase in the seminal plasma, as a result of which a part of 6-phosphofructose is converted to 6-phosphoglucose before dephosphorylation. Supporting evidence for the conclusion that monophosphofructose rather than diphosphofructose, is the substrate immediately responsible for the liberation of seminal fructose, has been provided by Bouchilloux and Menager (1952) who found that the semen of both ram and bull contains two phosphomonoesterases, with pH optima at 9·4 and 4·8, respectively, but that it lacks a specific fructosediphosphatase.

5-*Nucleotidase*

This enzyme was discovered in human semen by Reis (1937, 1938, 1940) and shown to dephosphorylate muscle adenylic acid (adenosine-5′-phosphoric acid) and inosinic acid (inosine-5′-phosphoric acid) but not adenosine-3′-phosphoric acid or adenosine triphosphoric acid. Bull seminal plasma is particularly rich in 5-nucleotidase, the seminal vesicles being the main source of the enzyme (Mann, 1945*a*, 1947). Bull seminal plasma or the vesicular secretion itself, act several hundred times more efficiently on muscle adenylic acid than on β-phosphoglycerol; from 160 μg. P added as sodium adenylate to 0·001 ml. bull seminal plasma, up to 140 μg. P are liberated as orthophosphate during 1 hour's incubation at 37°.

The 5-nucleotidase of bull seminal plasma has been purified about fifty-fold by Heppel and Hilmoe (1951*a*). The purified enzyme has a pH optimum at 8·5, and its activity is enhanced by the addition of magnesium ions but inhibited by fluoride (0·01M) and by

borate buffer (0·08M) to the extent of 73 and 85% respectively. On the basis of tests with numerous phosphorylated compounds, it may be safely concluded that 5-nucleotidase is an enzyme which acts specifically on substrates containing the ribose-5-phosphate moiety. The purified enzyme splits rapidly ribose-5-phosphate but not ribose-3-phosphate; it is active towards adenosine-5′-phosphate, inosine-5′-phosphate, uridine-5′-phosphate and cytidine-5′-phosphate but inactive towards both adenosine-3′-phosphate and adenosine-2′-phosphate. It also dephosphorylates nicotinamide mononucleotide (nicotinamide ribose-5′-phosphate); this incidentally explains an early observation of ours, that bull seminal plasma decomposes cozymase, with slow liberation of inorganic phosphate.

Pyrophosphatase

Bull seminal plasma contains an enzyme which hydrolyses inorganic pyrophosphate to orthophosphate but differs from the pyrophosphatase of yeast. Seminal pyrophosphatase can exert its maximal activity in the absence of magnesium ions and is not inhibited by increased substrate concentration; it has a sharp optimum at pH 8·6 (Heppel and Hilmoe, 1951*b*).

Enzymic hydrolysis of adenosine triphosphate

In addition to adenosine-triphosphatase (ATP-ase) in the spermatozoa there are also ATP-splitting enzymes in the seminal plasma (Mann, 1945*a*; MacLeod and Summerson, 1946). When adenosine triphosphate is acted upon by bull or human seminal plasma, all three phosphate groups are set free as orthophosphate. The mechanism of this reaction has been investigated by Heppel and Hilmoe (1953) who by fractionation procedures obtained three distinct ATP-ases, none of them, however, completely free from 5-nucleotidase.

One of the enzymes, named the 'pyrophosphate-forming ATP-ase', catalyses the reaction

$$\text{Adenosine triphosphate} + H_2O \longrightarrow \text{Pyrophosphate} + \text{Adenosine-5′-phosphate}$$

It is relatively heat-stable, has a pH optimum at 8·4–8·6 and requires neither calcium nor magnesium ions for activation. The

remaining two ATP-ases, designated respectively as 'acid' and 'alkaline', produce orthophosphate; the 'acid' ATP-ase has a pH optimum at 5·7–6·0, requires magnesium, is inhibited by calcium, and can be inactivated completely by heating for 20 min. at 60°; it does not act on β-phosphoglycerol; the 'alkaline' ATP-ase is more heat-resistant, has a pH optimum at 8·4–8·8, and is stimulated by calcium and also by magnesium. Both these enzymes are active not only towards adenosine triphosphate but adenosine diphosphate as well.

Bull seminal plasma contains in 1 ml. about 80 units of acid ATP-ase, 130 units of alkaline ATP-ase, 40 units of the pyrophosphate-forming ATP-ase, and 2900 units of 5-nucleotidase. It remains for future studies to define the physiological significance of all these enzymes, particularly the 5-nucleotidase which is so characteristic of semen. Possibly, there is some link between them and other nucleolytic enzymes and they may well play a role in the metabolism of purine compounds in semen and reproductive organs. The occurrence of nucleases in human and sea-urchin semen (Zamenhof, Shettles and Chargaff, 1950; Mazia, 1949), the cozymase-destroying activity of bull seminal plasma (Mann, 1945*a*), the interesting findings on the presence of uric acid in bull semen (Barron and Haq, 1948; Leone, 1952), and the more recent demonstration of xanthine oxidase in the bull vesicular secretion (Leone, 1953), are but a few examples of problems in this field, which await further and more detailed study.

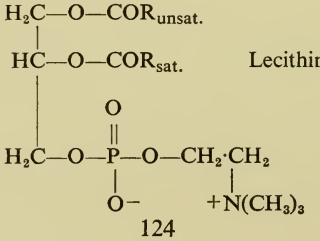
CHAPTER VI

Lipids and their Role in the Metabolism of Semen

Lipids in spermatozoa. The lipid capsule. Acetal phospholipids or plasmalogens. Role of lipids in sperm metabolism. Lipids in the seminal plasma and male accessory gland secretions. 'Lipid bodies' and prostatic calculi.

Lipids in spermatozoa

THE first systematic analysis of lipids in spermatozoa was carried out by Miescher (1878, 1897) who also proved that the lipids are concentrated chiefly in the sperm-tails. His analytical results showed that the ether-extractable material obtained from salmon spermatozoa is composed of about 50% lecithin, 14% cholesterol and 35% fat, and that by far the greatest part of this material is derived from the sperm-tails where lecithin accounts for 31·83%, fats and cholesterol for 26·27%, and protein for the remaining 41·90% of the organic contents. This led Miescher to conclude that the sperm-tails resemble in their composition the grey matter of the nervous system, and in a letter to W. Hiss he wrote: 'The more I deal with the tails, the more probable it appears to me that we have before us essentially the chemical type of the non-medullated nerves, that is the axis cylinders.' Subsequent investigations by Mathews (1897) and Sano (1922) on the sperm of herring, salmon, porgy and cod-fish, confirmed the presence of lecithin and revealed at the same time the presence of small quantities of certain other lipids, including cephalin and sphingomyelin.



Lecithin

When air-dried salmon roe is ground and extracted with pentane in a Soxhlet apparatus, a yellow oil is obtained which is practically free from phospholipids; it requires further treatment with ethanol or methanol for the phospholipids to be extracted from the sperm. In this way, for example, 12·5% glyceride in the oily fraction, and 6·2% phospholipid in the alcoholic fraction, was obtained from the roe of the sockeye salmon, *Oncorhynchus nerka* (Halpern, 1945). On the basis of this observation, it has been suggested that the phospholipids occur in the spermatozoa in a firmly bound state, presumably in the form of lipoproteins. A substantial portion of the unsaponifiable material extracted from fish sperm by fat solvents consists of cholesterol which accounts, on the average, for 2·2% of dried fish spermatozoa (Schmidt-Nielsen and Sundsvold, 1943).

The high content of lipids in spermatozoa is equally characteristic for fishes as for other animals. Sea-urchin spermatozoa are well known to contain a large reserve of lipid material, shown by Mathews (1897) to include both lecithin and neutral fat. The content of phospholipids in the sperm of *Echinus esculentus* is about 5·5% of the dry weight of spermatozoa (Rothschild and Cleland, 1952). The seminal lipids of *E. esculentus* have been analysed more recently by Cardin and Meara (1953). The material obtained by extraction of 1·2 l. of semen with acetone and light petroleum, consisted of 13·6% neutral fat, 32·9% free fatty acids, 26·0% phospholipids, 9·2% sterols and 18·3% of other unsaponifiable matter. The component fatty acids of the non-phospholipid fraction included a low proportion (10·1%) of saturated acids and a high proportion of unsaturated acids with 18*C* (30·4%) 20*C* (45·1%) and 22*C* (12·3%). The phospholipid fraction had a ratio of N : P=1·4 : 1, and must have therefore, consisted of a mixture of monoaminophosphatides and diaminophosphatides.

Early analyses of lipids in bovine epididymal sperm were carried out by Koelliker (1856) who found that over 12% of the dried material is ether-extractable. About half of this content was later shown by Miescher (1878) to consist of lecithin. In a study of the lipid content of bull sperm, Zittle and O'Dell (1941*a*) have extracted washed epididymal spermatozoa successively with ethanol, ether, acetone and petroleum ether, and found 13% of lipid material; when the procedure was repeated with spermatozoa disintegrated by sonic

treatment, more lipid was found in the tails (23%) than in the mid-pieces (6%) or heads (7%).

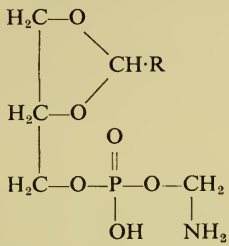
The lipid capsule

It is probable that the high lipid content of spermatozoa is due largely to the lipid 'sheath' or 'capsule' which encloses the sperm cell. So it would seem at any rate, from histochemical studies, including the extensive investigation of Popa and Marza (1931) who described the so-called *manteau lipidique* in the spermatozoon of man, dog, bull, ram, boar, rabbit, guinea-pig and cock. The lipid capsule is presumably of importance to the spermatozoa in their function, perhaps to ward off the effects of the acid vaginal milieu as has been suggested by Redenz (1924). It appears to consist largely of a lipoprotein complex which is fairly soluble in aqueous solvents. In the case of mammalian spermatozoa, this complex has been extracted with a 0·14M solution of sodium chloride at pH 9 (Dallam and Thomas, 1952). In the middle-piece lipids were shown to be associated with the 'spiral body' which surrounds the axial protoplasmic thread, and is derived from the mitochondria of the spermatids (Wislocki, 1950; Brown, 1952).

Acetal phospholipids or plasmalogens

An interesting feature of the sperm cell is a characteristically high content of acetal phospholipids or plasmalogens. Feulgen and Rosenbeck (1924) while studying the 'nucleal' reaction of human spermatozoa, noted that when fresh smears of human semen were treated with Schiff's fuchsin-sulphurous acid reagent, the middle-pieces and tails, though devoid of nuclear material, stained strongly. This observation was followed by a demonstration that cells in general contain in their protoplasm some material which stains diffusely with Schiff's reagent, but differs from nucleoproteins by its solubility in ethanol. The name 'plasmal' was bestowed upon this material, which was shown in subsequent investigations by Feulgen and his co-workers to arise from 'plasmalogen', a group of peculiar phospholipids widely distributed in tissues, and distinguished by the presence of higher fatty aldehydes in place of the usual fatty acids. The plasmalogen isolated by Feulgen and Bersin (1939) from beef muscle was identified as an acetal of glycerylphosphorylcolamine.

The two principal fatty aldehydes in plasmalogens are palmitic and stearic aldehydes but other fatty aldehydes were also reported (Feulgen, Boguth and Andresen, 1951). Crystalline acetal phospholipids were prepared from beef brain, and shown to belong to the α-series (Thannhauser, Boncoddo and Schmidt, 1951).



Acetal α-phospholipid

The plasmalogen content of bull semen as determined by Boguth (1952) was found to vary from 30 to 90 mg./100 ml.; of this about two-thirds is present in the sperm and one-third in the seminal plasma. The volume taken up by sperm in bull semen is comparatively small, about 10%; it would seem therefore, that the concentration of the acetal phospholipids in the spermatozoa themselves must be of the order of 200–600 mg./100 g. fresh weight, or 3×10^{-10} mg. per cell.

Heptacosane

The list of interesting chemical substances which occur in semen was extended again when in 1941 Wagner-Jauregg reported on the isolation of the hydrocarbon heptacosane from human semen. An alcoholic extract obtained from 18 litters of semen formed, upon concentration *in vacuo*, a solid residue which was extracted first with 1·5 l. acetone, and next with 2 l. of a mixture of equal amounts of ethanol and ether. On purification, the acetone-soluble fraction yielded some crystalline material which melted at 57–60° and consisted, in all probability, of palmitic and stearic acid. The ethanol-ether soluble fraction formed on standing a crystalline precipitate containing 1 g. of heptacosane, $CH_3(CH_2)_{25}CH_3$, which on recrystallization showed the required melting point, 59·5°. The isolation of heptacosane has previously been achieved from plant material. It is also known to be associated in a characteristic manner with beeswax.

So far, however, the only instance other than semen, where heptacosane has been shown to occur in the human body, is in the urine of pregnant women. It is absent from the urine of men and of non-pregnant women, and is devoid of oestrogenic activity. Nothing is known about the origin or function of seminal heptacosane. Should future investigations, however, show that heptacosane in semen is involved in the metabolism of fatty acids or aldehydes, then its fate would be analogous to that of plant hydrocarbons which are well known to be associated with the metabolism of fatty acids, aldehydes and alcohols in plants.

Role of lipids in sperm metabolism

The functional aspects of lipid metabolism in spermatozoa have been the subject of investigations by Lardy and Phillips (1941*a*, *b*; 1945). To begin with, these authors confirmed the observation originally made by Redenz (1933) that, in contrast to whole semen which can be stored successfully both anaerobically and aerobically owing to the presence of glycolysable carbohydrate in the seminal plasma, bull spermatozoa separated from the seminal plasma by centrifugation and washing, can survive only in the presence of oxygen. From this they inferred that when the spermatozoa are deprived of sugar, they begin to oxidize aerobically some of their own intracellular constituents as a source of energy for motility. To detect the oxidizable substrate, sperm samples were analysed when fresh and after periods of storage; it was then found that a period of aerobic incubation of bull spermatozoa caused a significant decrease in the content of lipid phosphorus accompanied by an increase in the acid-soluble phosphorus. But when glucose was added to the washed sperm, the decrease in the phospholipid content of spermatozoa was very slight, an indication perhaps, of a preference by spermatozoa for the glycolytic mechanism as a source of energy (Table 18). It was also found that certain phospholipids prepared from egg-yolk, liver and soya bean, effectively maintained the oxygen uptake and motility of washed sperm suspensions under aerobic conditions; however, on the addition of sugar to the sperm suspension, the phospholipids no longer produced an effect on either respiration or glycolysis.

In bull semen as ejaculated, with its large reserve of readily

TABLE 18. *Changes in the phospholipid content of bull spermatozoa in presence and absence of sugar* (Lardy & Phillips, 1941*a*)

(Bull spermatozoa freed from seminal plasma by centrifugation, then diluted with Ringer-phosphate solution to the original volume of semen, and incubated at room temperature.)

Medium	Phospholipid content	
	Original (mg. P/ml.)	After 10 hr. incubation (mg. P/ml.)
Ringer-phosphate	0·38	0·24
Ringer-phosphate+0·04M-glucose	0·39	0·37

glycolysable material in the form of fructose, the share of phospholipids in sperm metabolism is probably small. But in the epididymis, where glycolysable sugar is unavailable, Lardy and Phillips ascribe great importance to the phospholipids as a source of oxidative energy. The mechanism of utilization of this reserve is held to involve hydrolytic cleavage of phospholipids followed by an oxidation of the fatty acid portion via the citric acid cycle, and coupled with aerobic phosphorylations. According to Lardy, Hansen and Phillips (1945), the aerobic metabolism of phospholipids in the bovine epididymal spermatozoa is accompanied by an uptake of inorganic phosphate, and the formation of a phosphate ester which is hydrolysed in 7 min. by N-HCl at 100°, and thus resembles adenosine triphosphate.

The ability to utilize phospholipids as a source of aerobic energy extends to the spermatozoa of lower animals, notably those of the sea-urchin. One of the main differences between mammalian and sea-urchin semen is that the latter contains no glycolysable material in the seminal plasma. The possibility that sea-urchin spermatozoa which have been shed into sea-water, survive at the expense of energy derived from the oxidation of intracellular carbohydrate such as glycogen, also appears remote, in view of the very low content of glycogen-like material in the sperm cells (Stott, 1930; Rothschild and Mann, 1950). On the other hand, according to Rothschild and Cleland (1952), the content of intracellular phospholipids which in fresh sperm of *Echinus esculentus* is 5·5% of the dry weight or 4·14 mg. (0·165 mg. P) per 10^{10} sperm cells decreases in the course of aerobic incubation of sperm suspensions in sea-water, at an average

rate of 0·787 mg./10^{10} sperm cells/7 hr. The oxygen uptake recorded during the same period is 1·45 ml. O_2/10^{10} sperm, which if sustained exclusively by phospholipids, would require the disappearance of 0·906 mg. of phospholipid. On the basis of these observations Rothschild and Cleland conclude that the principal source of energy required for the movement of sea-urchin spermatozoa is derived from the oxidative breakdown of phosphilipids located mainly in the middle-piece of the sperm cell.

Lipids in the seminal plasma and male accessory gland secretions

Apart from the lipids which form a part of the sperm structure, there is also some lipid material in the seminal plasma. The bulk of the 'bound choline', however, does not consist of phospholipids but occurs in the form of acid-soluble phosphorylated derivatives of choline (see p. 170). The lipid of the human seminal plasma origin-

TABLE 19. *Lipids of the human prostatic fluid and seminal plasma* (*mg.*/100 *ml.*) (Scott, 1945)

(No. indicates the number of studied specimens.)

	Prostatic fluid				Seminal plasma			
Lipid fraction	No.	High	Low	Aver-age	No.	High	Low	Aver-age
Total lipid	10	310	260	286	10	206	166·6	185·5
Total phosphatide	10	225	144·2	179·8	10	132·6	47·8	83·5
Moist ether-soluble phosphatides	10	135	81·6	107	10	96	36·2	56
Lecithin	10	0	0	0	10	0	0	0
Cephalin	10	135	81·6	107	10	96	36·2	56
Moist ether-insoluble phosphatides	10	90	62·6	72·8	10	36·6	11·6	27·5
Total cholesterol	10	105	62	80	10	120	70	103

ates chiefly from the prostatic fluid. Moore, Miller and McLellan (1941) analysed twelve specimens of human prostatic secretion and found up to 9·5 mg. lipid phosphorus per 100 g. fluid, with an average of 2·7 mg. P/100 g. or 67·5 mg. phospholipid/100 g. Scott (1945), whose analytical results are shown in Table 19, found an average content of 286 mg./100 ml. of 'total lipid' and 179·8 mg./100 ml. of phospholipid, in the human prostatic secretion;

and 185·5 mg./100 ml. of 'total lipid' and 83·5 mg./100 ml. of phospholipid, in the seminal plasma. However, he was unable to detect lecithin either in the prostatic fluid or in the seminal plasma. In both instances, two-thirds of the phospholipid consisted of an ether-soluble choline-free phosphatide, probably identical with cephaline, the rest being some other, ether-insoluble material. Scott found little neutral fat in either the prostatic secretion or the seminal plasma, the sum of phospholipids and cholesterol accounting for practically the entire 'total lipid'. The content of 70–120 mg. total cholesterol/100 ml. seminal plasma recorded by Scott, is below the cholesterol value for human blood plasma; a similar figure, 80 mg./100 ml., has been reported earlier by Goldblatt (1935*a*).

'Lipid bodies' and prostatic calculi

In many species, the seminal plasma contains small globules, droplets or granules, sometimes called the 'lipid bodies'. In man, dog, cat, and rabbit, they are derived chiefly from the prostatic secretion but in certain species they occur also in the seminal vesicle secretion (Prévost and Dumas, 1824; Pittard, 1852). The globules of the human prostatic secretion are referred to by Sir Henry Thompson in his famous prize essay on the *Diseases of the Prostate* (1861), as 'small yellowish bodies, in appearance sometimes granular, sometimes homogeneous, about the size of red blood corpuscles, but not so uniform, being from about 1/5000 to 1/2500 of an inch in diameter' and exhibiting 'considerable refractive power nearly so much as to give them a resemblance to oil globules'. The occurrence of similar elements in the prostatic secretion was later observed by Fuerbringer (1881, 1886) who coined for them the name '*Lecithinkörnchen*'. These 'lecithin granules' or 'lecithin bodies' which Fuerbringer regarded as responsible for the normal opalescence and milky appearance of the prostatic fluid, have since been re-examined on several occasions, mostly by means of histological methods. Chemical analysis however, failed to corroborate the presence of lecithin in these particles. Other curious structures which according to some authors are closely linked with the appearance of 'lipid bodies' in the human seminal plasma, are certain larger bodies known as 'colostrum corpuscles', 'corpora amylacea' and 'prostatic calculi'. The colostrum corpuscles, frequently met with in the

human prostatic secretion, are macrophages packed with masses of lipid granules which stain strongly red with eosin. The corpora amylacea are small, soft, concentrically laminated spheroidal bodies, pale yellow to dark brown in colour, frequently, though not invariably, doubly refractile. They are usually located in the larger ducts and acini of the prostate and are probably made up of desquamated epithelial cells and prostatic secretion. They have been shown to contain some cholesterol but according to Moore and Hanzel (1936) the double refraction may be due to certain purines, decomposition products of nucleoproteins, and not to lipids. The prostatic calculi are ordinarily not more than a few mm. in diameter but occasionally they may replace the whole prostatic parenchyma. They are firm, calcified bodies, the basic structure of which, except for size and infiltration by calcium salts, is apparently the same as that of the corpora amylacea (Moore, 1936). Wollaston (1797) described them as composed of 'phosphorated lime in the state of neutralization, tinged with the secretion of the prostate gland'. He was also the first to show that they are not urinary products. In recent times, the chemical composition of prostatic calculi has been investigated by Huggins and Bear (1944); a considerable proportion of the prostatic stones was inorganic and consisted of calcium and magnesium phosphates and carbonates but there was also some 21% of organic matter composed of protein, citrate and cholesterol (Table 20).

Although characteristic of the human prostate, corpora amylacea are also found elsewhere, particularly among insectivores where their production is considered to be one of the chief secretory

TABLE 20. *Chemical analysis of prostatic calculi* (Huggins, 1947)

(Stones from 6 men; average values expressed in % of dry powdered material.)

Ash	79·00
Calcium	30·30
Magnesium	2·89
Phosphorus as PO_4	55·50
Carbon dioxide	1·27
Protein (N×6·25)	8·07
Citric acid	2·30
Cholesterol	7·13

functions of the prostate gland (Hopkins, 1911; Eadie, 1948*a*, *b*). They do not seem to occur either in the dog or the rat. The total lipid content of the dog prostatic fluid ranges from 30 to 40 mg./100 ml. (Huggins, 1947) and the lipid phosphorus from 1·1 to 2·2 mg. P/100 ml. (Moore *et al.*, 1941). There exists a condition known as the 'benign prostatic hypertrophy', which is common to dog and man. In the dog, however, this condition is not associated with the occurrence of corpora amylacea or any other spheroidal nodules but consists of cystic hyperplasia (Huggins and Clark, 1940). In the bull, lipid-laden cells form a highly characteristic component of the seminal vesicle epithelium, and the cavities of the tubules in the seminal vesicles contain an abundance of eosinophilic granular secretion (Mann, Davies and Humphrey, 1949). The extent to which organs other than the seminal vesicle and prostate, contribute to the lipid or sterol content of semen, has not been hitherto studied in much detail. In this connection, however, an interesting observation of Ward and Moore (1953) deserves to be mentioned, concerning the occurrence of 7-dehydrocholesterol in the preputial gland and epididymis of rat.

CHAPTER VII

Fructose and Fructolysis

Fructose as a normal constituent of semen. Species differences. Site of formation. Seminal fructose as an indicator of male sex hormone activity; the 'fructose test' and its application to certain problems of sex endocrinology. Role of hypophysis. The relationship between blood glucose and seminal fructose. Effect of malnutrition. The enzymic mechanism of fructose formation. Anaerobic and aerobic utilization of carbohydrate by spermatozoa. Pasteur effect and the 'metabolic regulator'. Intermediary reactions in sperm fructolysis and the role of phosphorus-containing coenzymes.

THERE has been little precise knowledge about fructose (laevulose) in man and higher animals except the evidence of its occurrence in certain embryonic fluids and in metabolic dysfunctions like fructosuria. The presence of a laevorotatory constitutent in foetal fluids was first noted by Claude Bernard (1855) but its chemical identity was not recognized until some time later when it was shown that fructose was a normal constituent of allantoic and amniotic fluid, foetal blood and the urine of new-born animals (Majewski, 1858; Grüber and Grünbaum, 1904; Paton, Watson and Kerr, 1907; Langstein and Neuberg, 1907; Orr, 1924; Cole and Hitchcock, 1946; Bacon and Bell, 1948; Hitchcock, 1949). More recently, the source of foetal fructose was traced to the placenta (Huggett, Warren and Warren, 1951). So far as adult man is concerned, it was believed that in general the occurrence of fructose is restricted to pathological conditions; fructose has been demonstrated in transsudates, and in the urine of diabetics and persons suffering from the peculiar metabolic disorder known as 'spontaneous fructosuria', the aetiology of which remains obscure. In the normal human or animal organism, fructose has been found to be utilized chiefly after enzymic conversion to glucose and glycogen; liver, kidney and the gastro-intestinal tract were shown to be the main sites of this process (Oppel, 1930; Bollman and Mann, 1931; Stewart and Thompson, 1941; Deuel, 1936; Reinecke, 1944).

Thus, in the light of the evidence available until relatively recently, it seemed rather improbable that in the normal, fully-developed mammalian organism, fructose could occupy a place on the list of 'animal carbohydrates', or that any specific function could be assigned to this sugar.

Fructose as a normal constituent of semen

Since the early researches on mammalian semen by McCarthy, Stepita, Johnston and Killian (1928), Ivanov (1931), Huggins and Johnson (1933) and other pioneers in the field of semen biochemistry, it was known that in several species, including man, a reducing and yeast-fermentable sugar is normally present in semen, the concentration of this sugar exceeding by far that of glucose in blood. However, up to 1945, in the extensive literature dealing with the subject of seminal sugar, this substance has been described either as glucose or simply as the reducing sugar of semen (Killian, 1933; Bernstein, 1933; Goldblatt, 1935*a*; Shergin, 1937; McKenzie, Miller and Bauguess, 1938; Davis and Cole, 1939; Moore and Mayer, 1941; MacLeod and Hotchkiss, 1942; Salisbury and VanDemark, 1945), and the only reference to a probable occurrence of fructose in semen is found in an early paper by Yamada (1933) who in a general survey of human tissues and body fluids carried out numerous fructose determinations by means of a colour reaction with the drug 'cryogenine'; of course, like so many colour tests, this reaction by itself cannot be regarded as specific for fructose, since it gives a positive result not only with fructose but also with other ketoses, nor does it distinguish between free fructose, that is D(−)-fructopyranose (formula in Fig. 14), and bound fructose, i.e. fructofuranose, such as occurs for example, in the various phosphofructoses.

In 1945, in the course of studies on the metabolism of semen, the seminal sugar was isolated for the first time and identified by chemical methods as free D(−)-fructose (Mann, 1945*b*; 1946*a*, *b*, *c*). The actual final isolation was accomplished with a 120 ml. sample of bull semen representing some thirty pooled bull ejaculates, which were collected within a twelve-hour period by the various Centres for Artificial Insemination of Cattle in England, and immediately despatched to Cambridge. The chemical procedure involved the following steps: (*a*) the preparation of methylphenyl-fructosazone, a

crystalline compound which has been shown by Neuberg (Neuberg, 1902, 1904; Neuberg and Strauss, 1902; Langstein and Neuberg, 1907; Neuberg and Mandl, 1946) to be one of the few chemical derivatives by means of which fructose can be identified and distinguished from glucose and from other closely related sugars; (*b*) the purification of seminal fructose up to the point when it reached the specific optical activity of pure crystalline fructose: $[\alpha]_D^{20^\circ} = -92{\cdot}2^\circ$; (*c*) the demonstration that fructose occurs in the semen in free form and that it accounts for the whole of the yeast-fermentable carbohydrate which yields 'ketose reactions' with resorcinol (Seliwanoff, 1887; Roe, 1934), diphenylamine (Ihl, 1885) and similar colour-producing substances (Pinoff, 1905; Thomas and Maftei, 1927; Pryde, 1946); (*d*) proof obtained with the highly specific enzyme, glucose oxidase, that in semen glucose is either absent or present in mere traces.

On the basis of the above findings, which excluded the presence of glucose, bound fructose, and other ketoses, a rapid colorimetric method has been developed by means of which it is possible to determine accurately the fructose content of semen; 0·05–0·1 ml. suffices for analysis of human, bull, ram or rabbit semen (Mann, 1948*a*, *b*; 1952).

Species differences

The following mammalian species have been found to contain fructose in semen: man, bull, ram, boar, stallion, goat, opossum, rabbit, guinea-pig, rat, mouse, hamster (Mann, 1949). Among the lower animals, fructose was found in the semen of an elasmobranch (the dogfish, *Scylliorhinus caniculus*) and in the reproductive organs of the male (but not female) grasshopper, *Locusta migratoria* (Humphrey and Mann, 1948; Humphrey, 1949). In this connection it is worthwhile to recall the occurrence of fructose in the haemolymph of the larvae of another insect, *Gastrophilus intestinalis* (Levenbook, 1947).

There are, however, considerable quantitative differences between the various species. In the bull and goat, for example, the concentration of fructose in semen sometimes reaches a level of 1000 mg./100 ml., but in the boar and stallion it seldom exceeds 50 mg./100 ml. Human semen occupies an intermediate position as

can be seen from Tables 4 and 5, which include values for fructose in several species. But, when comparisons are made between a species with fructose-rich semen (bull) and one notoriously poor in seminal fructose (boar), it must not be forgotten that the volume of a single boar ejaculate is almost a hundred times that of a bull, so that in effect, a single ejaculate of either species contains about the same absolute amount of fructose. There are species, however, in which fructose is altogether absent from semen or present only in traces, and it is through the study of these animals that we may hope to gain insight into the problem of alternative sugars in semen. Cock semen for example, has no fructose or a negligible amount only, but it contains a certain amount (20–100 mg./100 ml.) of anthrone-reactive material of which a variable fraction disappears on oxidation with glucose oxidase and must therefore, be identical with glucose (Mann and Hancock, 1952). Rabbit semen, unlike that of bull, ram and man, contains occasionally an appreciable admixture of glucose in addition to fructose (Mann and Parsons, 1950).

Site of formation

The reason for the conspicuous species differences in the concentration of fructose as well as the individual fluctuations (Table 4 and 5), is the fact that fructose is a product not of the testes, but of the male accessory organs of reproduction, principally the seminal vesicles (Mann, 1946*b*). Naturally, the highly variable anatomical characteristics of these glands such as their size, actual storage capacity, and secretory ability, are important factors which determine the final output of fructose in the ejaculate (Fig. 4). All these considerations are pertinent to studies of human semen because of the exceptionally large individual variations in the secretory function of the seminal vesicles and their rather small storage capacity which explains why the collection of consecutive ejaculates within a few days, usually yields samples with a conspicuously low level of fructose. It appears that a time interval of about two days is required to replenish the store of fructose in the vesicular secretion of man. Unlike in certain other mammals, the human seminal vesicle and vas deferens open into the urethra through a common channel known as the ejaculatory duct. Consequently, any obstruction at the

level of the ejaculatory ducts will prevent both fructose and spermatozoa from reaching the urethral canal. This fact has been aptly chosen as an aid to medical diagnosis by Young (1948, 1949) who described the case of a man in whom repeated semen analysis failed to detect fructose or sperm, although testicular biopsy revealed normal spermatogenesis; the case has been diagnosed as congenital bilateral aplasia of the vasa deferentia.

It must be also mentioned that though the seminal vesicles are the main source of fructose in the higher mammals, yet an additional small amount of this seminal sugar is derived from the ampullar glands (Mann, 1948*a*), and in some animals also from certain other glands. Thus, in the rabbit, fructose was located both in the glandula vesicularis (a structure corresponding to seminal vesicles) and in the ampullae, as well as in the prostate (Davies and Mann, 1947*b*). The rat provides an instance of particular interest, as in this rodent the seminal vesicles are free from fructose altogether; instead, fructose is found in the dorso-lateral prostate and in the so-called coagulating gland, a small organ immediately adjacent to the seminal vesicles proper, with which it shares a common peritoneal sheath (Humphrey and Mann, 1948, 1949).

Since fructose is produced by the accessory glands, and not the testes, it is not surprising that in whole fresh semen there is no direct proportion between fructose concentration and sperm density. On the contrary, both in man and in domestic animals, an inverse ratio between fructose and sperm concentration in semen is frequently met with; the simplest interpretation is that in a dense sample of semen the space occupied by the sperm cells is relatively larger, and the volume taken up by the fluid portion, i.e. the fructose-containing seminal plasma, correspondingly less. This factor has a direct bearing on the interpretation of laboratory examinations concerned with semen and male fertility or sterility. It explains, for instance, why a semen sample with a high content of fructose need not necessarily be one of good sperm quality, and furthermore, why it is possible to come across samples with a high fructose content but of low sperm density. In fact, some of our highest values for fructose so far recorded, were encountered in the semen of vasectomized, and thus completely azoospermic, individuals.

Seminal fructose as an indicator of male sex hormone activity; the 'fructose test' and its application to certain problems of sex endocrinology

The 'fructose test', originally described by Mann and Parsons (1947) and subsequently developed by Mann, Davies and Humphrey (1949), Mann, Lutwak-Mann and Price (1948) and Mann and Parsons (1950), is founded on the observation that the capacity of the accessory organs to produce fructose and, thereby, the actual level of fructose in the seminal plasma, reflects in a faithful manner the degree of testicular hormone activity in the male, and in this way provides an accurate indicator of endocrine testicular function. In experiments on rats and rabbits it was shown that seminal fructose disappears almost completely within two weeks after castration and also that the postcastrate fall in the level of fructose can be prevented, or, if already developed, fully restored, by the implantation of testosterone (Fig. 12). The effect is not limited to laboratory animals and analogous results were obtained with domestic animals such as the bull.

The test can be carried out in two ways, by the chemical analysis of the seminal fluid collected from an intact animal by means of an artificial vagina, or by the analysis of accessory organs of reproduction obtained from the experimental animal by dissection. The first method gives an opportunity to observe in the same animal the time-sequence of changes brought about by castration and hormonal treatment, and eliminates the sacrifice of the experimental animal. In the second procedure, on the other hand, the test can be used for a quantitative assay of male sex hormone activity in the whole body, isolated tissues, body fluids and hormone preparations; as an illustration, Fig. 13 gives a dosage-response curve obtained with coagulating glands of castrated rats which were injected for three weeks with known doses of testosterone propionate; following the last day of injections the rats were sacrificed, the coagulating glands dissected and used for fructose analysis.

Below are discussed some of the endocrinological problems to which an approach was made in recent years with the aid of the 'fructose test', applied either alone or in conjunction with the 'citric acid test', which depends on the relationship between the secretion

of citric acid by some of the accesory organs and the male sex hormone activity.

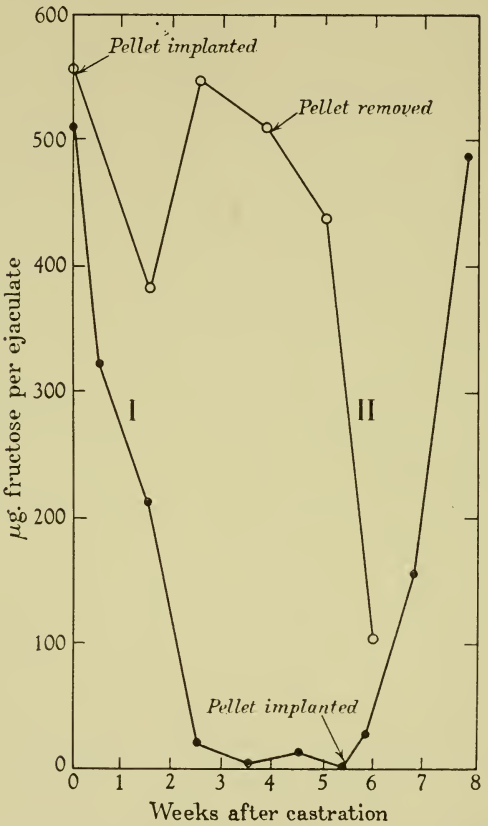


FIG. 12. *Post-castrate fall and testosterone-induced rise of seminal fructose in rabbit;* pellet: 100 mg. testosterone.
(Mann & Parsons, 1947)

(*a*) *Time relationship between the onset of secretory activity in male accessory glands and spermatogenesis.* In young rabbits (Davies and Mann, 1947*b*), rats (Mann, Lutwak-Mann and Price, 1948), bull-calves (Mann, Davies and Humphrey, 1949) and boars (Mann, 1954),

fructose and citric acid appear in the accessory glands at an early age, before there is any evidence of active spermatogenesis; since the secretion of both these substances depends upon the presence of the male sex hormone, it must be concluded that the hormone begins to function in the male body well in advance of the actual spermatogenesis. Thus, for instance, in bull-calves appreciable

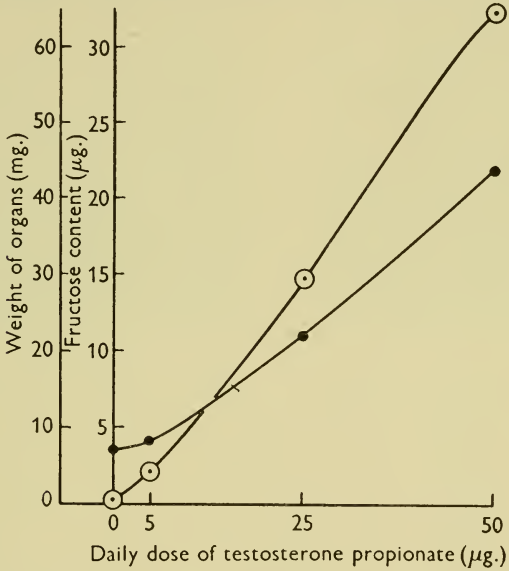


FIG. 13. *Dosage-response curves of testosterone propionate, using the coagulating glands of the rat;* ⊙——⊙, fructose (μg.); ●——●, weight of organs (mg.).

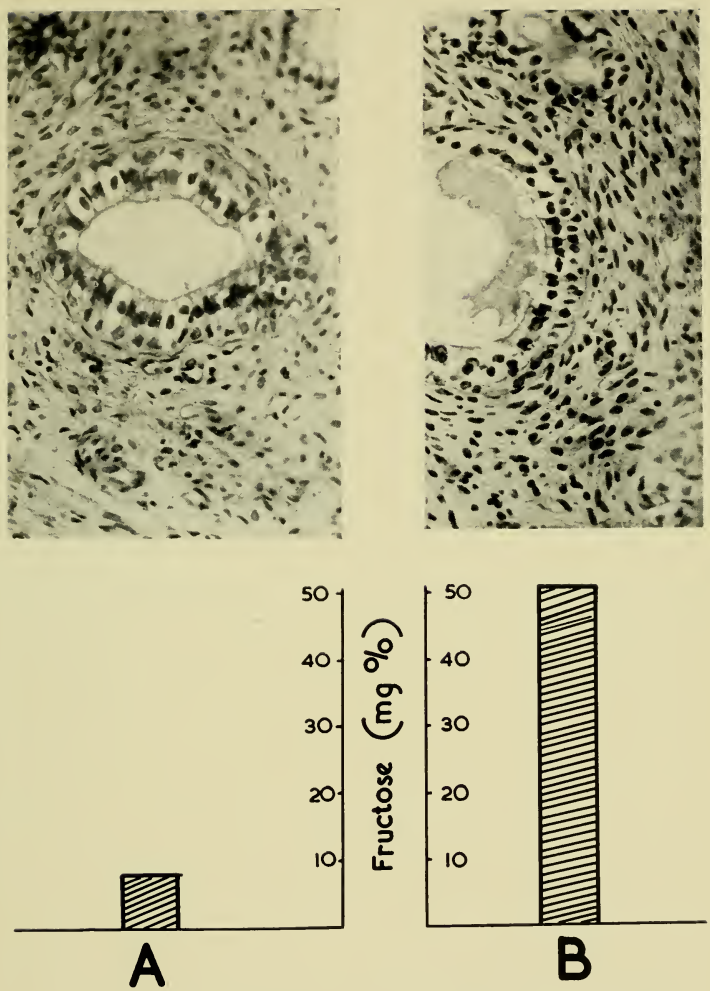
(Mann & Parsons, 1950)

amounts of fructose are found in the vesicular secretion already at the age of about four months, whereas the first mature spermatozoa appear nearly eight months later. One cannot, of course, rule out the possibility that the testicular hormone is active in the bull-calf even before the age of four months, but if so, then either its concentration is too small to produce a distinct response in the accessory organs, or else its action is countered by some other factors.

(*b*) *Effect of testosterone on the appearance of fructose in castrated*

animals. The following experiment was carried out by Mann, Davies and Humphrey (1949) at the Agricultural Research Council Field Station at Compton, in Berkshire. Six bull-calves were used. These were castrated when one to two weeks old, i.e. at an age prior to the appearance of fructose in the seminal glands. Seven months later two of the castrated calves received subcutaneous implants of 0·5 g. pellets of pure testosterone, whereas the remaining four were left untreated. After another four weeks all six animals were sacrificed and their seminal glands dissected out, weighed, and examined both chemically and histologically. The unused portions of the hormone pellets were recovered from the subcutaneous tissue of the two hormone-treated calves; their weights were 0·344 and 0·338 g. respectively, showing that the quantities of testosterone absorbed per month per animal were 0·156 and 0·162 g., respectively. Chemical analysis revealed the presence of considerable amounts of fructose in the seminal glands in response to the four weeks' hormone treatment (51 mg. fructose per 100 g. tissue or 5·3 mg. fructose per total gland), as against a negligible fructose content in the untreated castrates (8 mg. per 100 g. or 0·25 mg. per total gland). In comparison with and in contrast to the marked chemical difference, the evidence for the functional recovery in the seminal glands, as assessed by the histological examination, was practically imperceptible (Plate VI). In this way we were able to provide evidence that the early effects of testosterone treatment can be established far more convincingly by the large percentage-increase in the fructose content of the seminal gland secretion, than by means of histological methods which at this stage failed to show significant changes in the glandular tissue.

An investigation concerned with the response to testosterone was also made by Rudolph and Samuels (1949) on rats, and by Gassner and his co-workers (1952) on bulls. In castrated rats, a significant increase in the fructose content of accessory organs was noticed already ten hours after the injection of 1 mg. testosterone propionate. In bulls, fructose disappeared from ejaculates within two weeks after castration but injections of testosterone propionate, if given within four weeks after castration, led to a rapid return of fructose production to the pre-castrate level; yet, in spite of the fully restored fructose level, such seminal plasma, when added to washed



EFFECT OF CASTRATION AND TESTOSTERONE ON BULL SEMINAL VESICLES

Histological sections from a tubule (mag.×437), and the fructose content of seminal vesicle.

A. from a bull-calf castrated when three weeks old, and killed when nine months old.

B. from a bull-calf castrated when three weeks old, left untreated till eight months old, and then implanted with testosterone (0·5 g.); killed one month later, simultaneously with calf A.

spermatozoa obtained from a normal bull, was unable to support sperm metabolism to the same extent as plasma from normal i.e. non-castrated animals.

An interesting example of the application of the fructose test to problems of infertility in man has been provided by a study of four eunuchoid patients who responded to androgenic treatment with a highly significant elevation of fructose in semen (Landau and Loughead, 1951).

It seems probable that the fluctuations of fructose level in the semen of normal individuals may also be due, in part at least, to some periodic changes in the activity of the testicular hormone in the male body. Normal rats, injected with large doses of the male hormone invariably react by an increased level of fructose formation, well above that of non-treated controls. The effect is particularly striking with breeds of animals which exhibit a relatively low physiological level of fructose formation. In this connection, however, it is interesting to note that when injections of large doses of testosterone propionate into normal rats are continued to excess, e.g. 200 μg. daily for forty days, the state of overstimulation in the accessory organs is accompanied by a marked decline in the size of the testes; after seven weeks of such treatment the reduction in the weight of the testes is nearly 50% (Mann and Parsons, 1950). Injections of excessive doses of androgens are well known to produce harmful effects on the spermatogenesis in animals and in man (Moore, 1939; McCullagh and McGurl, 1939; Heckel, 1951).

In normal bulls, a dose of 100 mg. testosterone propionate, repeated three times weekly for six weeks, appears to produce only a very slight increase in the level of fructose in semen (Gassner, Hill and Sulzberger, 1952). However, according to another report, sexual excitation prior to service has a stimulating effect on the output of fructose in bull semen (Branton, D'Arensbourg and Johnston, 1952).

(*c*) *Hormone-induced formation of fructose in subcutaneous transplants from accessory organs.* Once the dependence of seminal fructose upon the activity of the male sex hormone had been established it was possible to enquire into the mechanism of this hormonal relationship. One of the problems to settle was the extent to which the process of fructose generation in accessory glands depends upon the

preservation of intact vascular and neural links. Insight into this matter was gained by the technique of subcutaneous transplantation, when it was demonstrated that small fragments of rat coagulating gland, about 1 mg. in weight, transplanted subcutaneously into normal adult male hosts, grew well and showed after some weeks of subcutaneous development a high content of fructose. Following castration of the hosts, the transplants lost their ability to form fructose but this was promptly restored by treatment with testosterone propionate. Perhaps the most remarkable fact in these experiments was that the growth of the grafts and their chemical secretory function occurred not only in male, but also in female hosts provided that the latter were treated with testosterone (Lutwak-Mann, Mann and Price, 1949).

Thus, for the first time the effect of the male sex hormone on fructose secretion was demonstrated in tissue fragments dissected from the male accessory organs and developing in complete isolation from the rest of the male generative system. Actually, the transplants had an even higher fructose content than the corresponding intact glands of the graft-bearing hosts, because unlike intact glands, the grafts lack a secretory outlet.

In another study, Price, Mann and Lutwak-Mann (1949, 1954) applied the transplantation technique, coupled with the chemical methods, to the problem of the androgenic activity of ovarian hormones in the female rat. Subcutaneous transplants of rat coagulating gland in female hosts were shown to secrete large quantities of fructose in response to injections of pregnant mare serum gonadotrophin. A series of thirty injections of twenty international units of equine gonadotrophin was given daily; at autopsy the ovaries of the female hosts were enlarged at least tenfold and covered with numerous follicles and corpora lutea. In these rats, gonadotrophin, through a stimulating action on the ovaries, raised the output of ovarian androgens to an extent which induced the secretion of fructose in transplants from the coagulating gland.

(*d*) *Effects of progesterone, stilboestrol and oestradiol.* The nature of the ovarian androgen responsible for the formation of fructose is unknown, but there are indications that it may be related to progesterone or to a product of progesterone metabolism. An inquiry into the androgenic value of progesterone showed that large

doses of progesterone injected into castrated male rats have a definite androgenic effect; it was calculated that the androgenic value of 25 mg. pure progesterone is slightly more than that of 0·005 mg. testosterone propionate (Price, Mann and Lutwak-Mann, 1949, 1954).

Whereas progesterone exhibits some androgenic activity, stilboestrol is endowed with the properties of an androgen-antagonist. The testosterone-catalysed secretion of fructose in the male accessory gland secretions of a rabbit can be suppressed very effectively by the subcutaneous implantation of 25 mg. stilboestrol (Parsons, 1950). In experiments in which a castrated rabbit received simultaneously implants of testosterone and stilboestrol, the latter prevented completely the production of fructose by the accessory organs.

It appears that in castrated bulls, small amounts of oestradiol dipropionate used together with testosterone, have a small but definite synergistic effect on the seminal vesicles and lead to a higher output of fructose in semen (Gassner, Hill and Sulzberger, 1952).

Role of hypophysis

The endocrine influence of the testes on the formation of fructose in accessory organs is integrated closely with the functioning of the anterior pituitary gland. Hypophysectomy, like gonadectomy, invariably results in a rapid decline in the level of fructose in the seminal plasma (Mann and Parsons, 1950). In the rabbit, for instance, a three to four weeks' period after castration or hypophysectomy alike, usually leads to complete disappearance of fructose so that an ejaculate collected by means of an artificial vagina three weeks

TABLE 21. *Effect of testosterone and pregnant mare serum gonadotrophin on the formation of fructose in rabbit prostate*

Rabbit	Weight of prostate (mg.)	Fructose content (μg./organ)
1. Normal	770	935
2. 3 weeks after castration	280	20
3. 6 weeks after castration and simultaneous implantation of testosterone (100 mg.)	1000	1220
4. 4 weeks after hypophysectomy	149	10
5. 6 weeks after hypophysectomy; for the last 4 weeks injected 200 I.U. PMS-gonadotrophin	210	395

after the operation contains no more than 20 μg. fructose, as compared with 500 to 1000 μg., before the operation. Both castrated as well as hypophysectomized animals promptly respond to the subcutaneous implantation or injection of testosterone with renewed secretion of fructose. The same happens if instead of testosterone pregnant mare serum gonadotrophin is injected into a hypophysectomized animal (Table 21).

The relationship between blood glucose and seminal fructose

In addition to the hormones of the testis and the pituitary gland, yet another organ, the pancreas, exerts a profound influence upon the level of fructose in semen. The effect is an indirect one, and is brought about by the action of insulin on the level of blood glucose which in turn governs the level of fructose in semen. The existence of a causal link between the blood sugar level and seminal fructose was studied at first in animals with experimental diabetes; later, however, it was also shown in diabetic man (Mann and Parsons, 1949, 1950).

In rabbits, experimental diabetes can be produced with alloxan; best results are obtained by injecting intravenously into a rabbit 75 mg. alloxan per kg. body weight, and repeating this dose one or two days later. Fig. 14 illustrates the course of such an experiment with a rabbit in which analyses of blood and semen were carried out regularly during a period of four months. At the outset of the experiment this animal had a blood glucose content of 100 mg. per 100 ml., and about 600 μg. fructose per ejaculate or 70 mg. per 100 ml. semen (fluid portion). However, following alloxan treatment, the rabbit developed severe glucosuria within two days and its blood glucose level rose to 350 mg./100 ml.; at the same time, the level of fructose in semen began to increase, until three weeks later there was 500 mg. glucose per 100 ml. blood, and 3500 μg. fructose per ejaculate or 320 mg. fructose per 100 ml. semen. When it was established that hyperglycaemia is followed by an increased concentration of fructose in semen, the effect of insulin was examined and it was found that the insulin-induced fall in blood glucose was followed by a reduction in the fructose content of semen; moreover, once the effect of insulin on blood glucose in the diabetic animal wore off, there was again an increase in seminal fructose.

Conditions similar to those in experimental diabetes seem to prevail also in man. In semen samples from diabetic patients we



FIG. 14. *Effect of diabetes and insulin on seminal fructose in rabbit.* The period of insulin treatment is indicated by arrows. Semen was collected weekly.

came upon fructose values which were well above the upper limit of normal variations; diabetic values ranged from 650 to 1230 mg. per 100 ml., and from 33·4 to 47·5 mg. per ejaculate. In a survey of 150 specimens of normal human semen, Harvey (1948) found

640 mg. per 100 ml. or 31·6 mg. per ejaculate to be the highest value. It is interesting to recall here that years ago Goldblatt (1935*a*) noticed a high reducing sugar value in human diabetic semen but attributed this to urinary glucose.

Effect of malnutrition

It has long been known that defective nutrition has a deleterious influence upon the male reproductive system. One of the earliest surveys of this problem is found in the monograph by Jackson (1925); this was followed by the work on degenerative changes in testes and sterility associated with vitamin A and E deficiency and in later years, by many other nutritional studies which helped to accumulate a wealth of information on this subject, fully reviewed on several occasions (Asdell, 1949; Burrows, 1949; Lutwak-Mann, 1951; Mason, 1949; Reid, 1949; Russell, 1948; Samuels, 1948; Walton, 1949). Most investigators in this field, however, particularly those concerned with problems of human fertility, were much more interested in the spermatogenic activity of the testicular tissue than in the function of the accessory organs of reproduction. It was, therefore, something of a departure when Moore and Samuels (1931) came forward with the demonstration that a few weeks of a diet deficient in vitamin B, or a quantitatively inadequate diet containing vitamin B, caused in male rats regressive changes in the accessory organs which, however, could be counteracted by the administration of testicular hormone or anterior pituitary extracts. They concluded that the primary lesion due to inadequate feeding was located in the pituitary gland and that as a result of the diminished hypophyseal activity the testes received insufficient gonadotrophic stimulus and were consequently, unable to produce the male sex hormone required for normal functioning of the accessory glands. A similar state of 'pseudo-hypophysectomy' was described by Mulinos and Pomerantz (1941) in rats as the result of a diet which was qualitatively adequate but halved in quantity; further supporting evidence was later provided by several groups of investigators (Pazos and Huggins, 1945; Goldsmith and Nigrelli, 1950; Grayhack and Scott, 1952). In certain animal species spermatogenesis was also shown to be affected by a vitamin B-deficient

diet (Marrian and Parkes, 1928; Dunn, Morris and Dubnik, 1947; Elson and Koller, 1948).

Lutwak-Mann and Mann (1950*a*, *b*, 1951) applied chemical methods to the study of changes brought about in the secretory function of rat accesory organs by vitamin B-deficiency and inanition, and found that in rats maintained for four weeks on a deficient diet the content of fructose and citric acid in the accessory glands was reduced to a castrate level. By treatment with testosterone propionate (0·2 mg. daily for one week) or with chorionic gonadotrophin (200 units every other day for two weeks), the secretory activity of the glands could be completely restored. A further example of the effect of an unbalanced diet on the process of fructose secretion was provided by Lutwak-Mann (1951) who found that a diet with excessive fat content, even though not protein- or vitamin-deficient, also caused regression in rat accessory organs. Mann and Walton (1953) made a study of the effect of underfeeding on the genital functions in the bull and found that, in contrast to the testes, the secretory function of the male accessory glands was markedly affected by underfeeding. The concentration of fructose and citric acid in the semen of the underfed bull decreased by 30% and 60% respectively, of the original levels. In the bull, however, unlike in the rat, the effects of malnutrition as well as the recovery after the animal has been transferred back to its normal diet, developed comparatively slowly.

The enzymic mechanism of fructose formation

The experimental evidence available at present brought out the essential, though as yet not fully understood, role of the testicular hormone in the formation of fructose by the secretory apparatus of the male accessory glands, and indicated that blood glucose is the precursor of seminal fructose. Further details of the mechanism whereby glucose is converted in the accessory gland tissue to fructose, were obtained from *in vitro* experiments; these showed that small amounts of fructose are formed as a result of incubation of minced accessory gland tissues with glucose, and that these tissues possess the entire enzymic system which can convert glucose to fructose (Mann and Lutwak-Mann, 1948, 1951*a*, *b*).

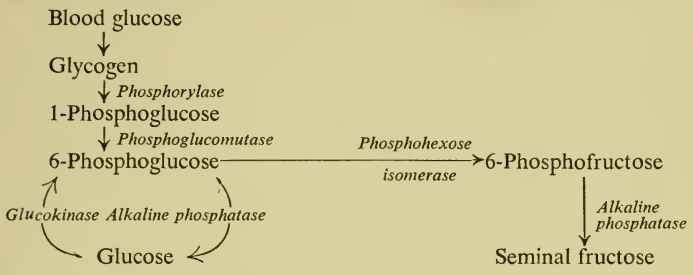
It is an established fact that certain phosphorylated derivatives of

fructose, such as 6-phosphofructofuranose (Neuberg ester) and 1 : 6-diphosphofructofuranose (Harden-Young ester), are formed as intermediary substances in the normal carbohydrate metabolism of muscle, liver and other animal organs. However, in the majority of animal tissues these phosphofructoses do not yield free fructose but are metabolized further to form pyruvic acid and lactic acid. In the semen, however, there are present in high concentration enzymes which belong to the group of phosphatases and include the 'alkaline' phosphatase; the latter capable of splitting a number of phosphohexoses, including 6-phosphofructose, 1-phosphofructose and 1 : 6-diphosphofructose, to phosphoric acid and free fructose. The alkaline phosphatase found in semen is derived from several accessory organs of reproduction but its principal source in higher animals is the seminal vesicle. Owing to this fact, the usual channels of carbohydrate metabolism are diverted in the vesicular tissue: phosphofructoses are not metabolized to lactic acid, as would be the case e.g. in muscle, but are dephosphorylated instead, so that free fructose is formed.

In extracts made from bull seminal vesicle tissue it is possible to demonstrate the following reactions. When 1-phosphoglucose is incubated, 6-phosphoglucose is formed through the action of phosphoglucomutase; next, part of 6-phosphoglucose is converted by phosphohexose isomerase into 6-phosphofructose. The equilibrium mixture of the two 6-phosphohexoses is acted upon by phosphatase and yields a mixture of free glucose and free fructose. Phosphohexose isomerase, together with alkaline phosphatase is also present in the seminal vesicle secretion and seminal plasma. Ram seminal plasma in particular, is a rich source of phosphatase active towards phosphorylated sugars (Table 17, p. 120).

The fact that whereas a mixture of glucose and fructose is the result of the phosphatase activity in the glandular tissue, yet, only one sugar, that is fructose, accumulates in the secretion, may have its explanation in a more effective re-utilization of glucose than fructose, by the glandular tissue itself. Thus, we have found that tissue slices from the rat coagulating gland can glycolyse anaerobically glucose at a much higher rate than fructose; this, in turn, may be due to the ability of the tissue to re-phosphorylate more effectively glucose to 6-phosphoglucose, than fructose to

6-phosphofructose. Such evidence as is at present available, derived from both *in vivo* and *in vitro* experiments, indicates that the enzymic reactions involved in the conversion of blood glucose to seminal fructose are as follows.



Anaerobic and aerobic utilization of carbohydrate by spermatozoa

The spermatozoa of the sea-urchin and certain other animals derive their energy for movement chiefly from respiratory processes; in contrast, the survival and motility of sperm ejaculated by animals with internal fertilization, such as mammals, is possible for most of them also in absence of oxygen, provided that the sperm cells remain in contact with seminal plasma. Mammalian spermatozoa possess only a negligible reserve of intracellular glycogen and depend therefore, under anaerobic conditions, on an extracellular source of energy. In species which contain fructose as a normal constituent of the seminal plasma, anaerobic fructolysis is the metabolic process which enables the spermatozoa to survive without oxygen. Should, however, the spermatozoa become separated from the seminal plasma by centrifugation and washing, they could not carry on anaerobically unless the seminal plasma were restored or replaced by glycolysable carbohydrate.

The stimulating effect of pure sugars on sperm motility has been noticed by some of the early investigators of semen. In 1931 Ivanov observed that dog spermatozoa suspended in an isotonic solution of glucose and phosphate retained their motility when the respiration had been abolished either by poisoning with cyanide or by replacement of oxygen with hydrogen. This observation is of particular interest in view of the fact that the dog has no seminal vesicles and

no fructose in the seminal plasma; the possibility, of course, must not be overlooked that there may be in dog semen some other substance of nutrient value to the spermatozoa. Redenz (1933) has shown that bull spermatozoa glycolyse glucose, fructose, and mannose to lactic acid, and that the presence of these sugars, but not that of sucrose, lactose, or glycogen, is beneficial to sperm motility. His findings were confirmed by others and it has since become an established fact that the metabolism of spermatozoa in several mammalian species including man, ram and bull, is predominantly of a glycolytic character (Ivanov, 1935; Shergin, 1937; Comstock, 1939; MacLeod, 1939, 1943*b*; Lardy and Phillips, 1941*a*; Moore and Mayer, 1941; Henle and Zittle, 1942; Ross, Miller and Kurzrok, 1941; Salisbury, 1946).

Spermatozoa obtained directly from the epididymis of a bull, ram, or boar, resemble washed ejaculated sperm in that they are incapable of survival under purely anaerobic conditions. While in the epididymis, the spermatozoa have no access to fructose and are immotile; the onset of motility coincides with their passage along the male genital tract and contact with the seminal plasma. The activating influence of fructose on previously immotile spermatozoa can be convincingly demonstrated in a simple manner. Fresh epididymal spermatozoa are suspended in bicarbonate-Ringer solution; two 'hanging drops', one of the suspension and another, a little further away, containing a 1% solution of fructose in bicarbonate-Ringer solution, are placed on the underside of a cover-slip; to observe the motility of the sperm under the microscope, the cover-slip is fixed to the top of a small glass chamber in which one can create anaerobic or aerobic conditions by passing through the chamber a gas mixture of 95% N_2–5% CO_2 or 95% O_2–5% CO_2. In the absence of oxygen, the spermatozoa can be seen to be almost completely immotile, but when the two drops are brought together the sperm movement begins and continues for a long time. Aerobically, the effect of fructose is less striking because oxygen induces endogenous respiration and this in itself provokes motility in epididymal spermatozoa. However, even in the presence of oxygen, fructose still has some influence owing to the process of aerobic fructolysis.

Under anaerobic conditions, the final product of sperm fructolysis, lactic acid, cannot be oxidized further. In the presence of oxygen,

however, the situation differs in that the rate of fructose utilization becomes smaller, and moreover, lactic acid undergoes further oxidation, thus providing an additional source of metabolic energy. It remains for further study to ascertain what type of carbohydrate metabolism predominates in spermatozoa during their existence in either the male or female genital tract. However, so far as *in vitro* studies are concerned, they show that lactic acid can be efficiently oxidized by spermatozoa even when the partial pressure of oxygen has been reduced to a level as low as that which normally prevails in animal tissues (Mann, 1951*b*); suitably diluted suspensions of ram spermatozoa show in presence of 1% O_2 a respiratory rate as high as in air, and lactate is capable of maintaining the oxygen uptake equally well in 1% as in 20%, oxygen.

Pasteur effect and the 'metabolic regulator'

It was said earlier that the spermatozoa obtained directly from the epididymis in some ways behave like suspensions of washed ejaculated sperm; as a matter of fact, however, these two types of sperm cells possess distinct characteristics (Henle and Zittle, 1942; Lardy, Hansen and Phillips, 1945). Washed epididymal bovine spermatozoa have a lower endogenous respiration than those in ejaculated bull semen. But if sugar is added or if the spermatozoa are removed from the epididymis after a period of storage in the refrigerator, then their oxygen uptake is distinctly higher. Moreover, on addition of sugar, epididymal spermatozoa produce lactic acid much more rapidly under anaerobic than aerobic conditions, whereas in ejaculated sperm the rate of glycolysis is not much higher in the presence than in the absence of oxygen. To account for these differences, the Wisconsin workers determined the rate of anaerobic and aerobic glycolysis as well as of oxygen uptake, in epididymal spermatozoa to which glucose was added, and calculated the 'Meyerhof oxidation quotient' which measures the Pasteur effect, that is the extent to which glycolysis is inhibited by oxygen. The average value for the Meyerhof quotient calculated from twelve experiments on bull epididymal sperm was 9·6, as against 5 recorded for ejaculated sperm (Lardy, 1952). This difference, according to Lardy, Ghosh and Plaut (1949), is due to the presence in bull spermatozoa of a 'metabolic regulator' which occurs in the epididymal

sperm in a 'bound form' but is released in an 'active form' after ejaculation. Continuing their study, these workers observed that heat-inactivated bull semen, or semen and testicular extracts heated with sodium hydroxide, increased the rate of aerobic fermentation of sugars by baker's yeast, without affecting markedly yeast respiration or anaerobic fermentation. The yeast-stimulating factor was extracted with carbon tetrachloride from alkaline hydrolysates of hog testes and obtained in the form of yellow coloured crystals which proved to be elementary sulphur (Ghosh and Lardy, 1952). Yeast reduces sulphur to H_2S which is probably the agent ultimately responsible for the stimulation of the aerobic fermentation. The identity of the yeast factor with sulphur was verified by reproducing the stimulating effect on yeast with pure rhombic sulphur. Sulphur as such, however, cannot be the sperm 'regulator' since it is without influence on the Pasteur effect in epididymal spermatozoa. On the other hand, a number of sulphydryl compounds such as cysteine, reduced glutathione and hydrogen sulphide have been found to stimulate the respiration and aerobic glycolysis of epididymal sperm and the possibility remains, that the 'metabolic regulator' is, in fact, a sulphydryl compound, liberated during ejaculation from the spermatozoa, with a sulphydryl group in labile form, which can be easily removed and oxidized to sulphur by alkaline hydrolysis.

The peculiar changes in the metabolic properties of spermatozoa during cold-storage of the epididymis, are equally in need of elucidation. A problem which also deserves further biochemical study is the 'ripening' phenomenon which takes place in the spermatozoa while they remain in the epididymis. Presumably, the metabolism of sperm in the epididymis is related in some as yet unknown manner to the structural changes associated with sperm maturation processes, such as the migration of the 'kinoplasmic droplet'.

Intermediary reactions in sperm fructolysis and the role of phosphorus-containing coenzymes

The ability of washed spermatozoa to convert into lactic acid equally well added fructose, glucose and mannose is due in all probability to the fact that the metabolic degradation of these three sugars is initiated by the same hexokinase-catalysed reaction with adenosine triphosphate.

Adenosine triphosphate (ATP, formula in Fig. 15) represents an intracellular constituent and a coenzyme of considerable importance in the economy of the sperm cell. An observation that a considerable proportion of the acid-soluble phosphorus in bull spermatozoa yields orthophosphate after 7 min. hydrolysis with N-HCl first suggested the presence of ATP (Lardy and Phillips, 1945). In the same year, the readily-hydrolysable phosphorus compound was isolated from ram spermatozoa and its identity with ATP established by chemical analysis (Mann, 1945*a*, *c*); the content of ATP in ram spermatozoa is 2·6–6·6 mg. labile phosphorus or 0·6–1·5 mg. of adenine amino-nitrogen per 100 ml. semen (see also Table 16). The occurrence of ATP in ram and boar spermatozoa has also been confirmed by Ivanov, Kassavina and Fomenko (1946) who found that the phosphorus compound which they purified from sperm induced contractions of muscle actomyosin threads in the same manner as ATP isolated from skeletal muscle. ATP was also found in sea-urchin spermatozoa (Rothschild and Mann, 1950), the concentration of ATP in the semen of *Echinus esculentus* resembling that found in the ram.

Spermatozoa, even after they have been repeatedly washed so as to remove the phosphatases present in seminal plasma, continue to exhibit a high phosphatase activity against ATP, and all evidence available at present points to sperm ATP-ase as the enzyme which is directly responsible for the supply of energy essential for normal motility and survival of the sperm cell. The losses due to utilization of ATP are made good by re-synthesis which takes place during the normal metabolism of spermatozoa, and any interference with intermediary enzymic reactions which renders the sperm cell incapable of breaking down or building up ATP, leads to a decrease in both metabolism and motility. Using ram spermatozoa as experimental material under a variety of conditions, we have found that a diminution in the content of ATP invariably coincides with impaired sperm motility (Mann, 1945*a*, *b*, *c*). Thus, for instance, in ram spermatozoa deprived of the fructose-containing seminal plasma by washing, ATP content as well as motility went down simultaneously on anaerobic incubation, but both ATP and motility could be preserved anaerobically in sperm suspensions provided with glycolysable material.

The activity of hexokinase, the enzyme which brings about the initial reaction between ATP and glycolysable sugar, can be demonstrated directly in washed spermatozoa (Mann, 1945*b*). If we add to a ram sperm suspension sugar (glucose, fructose or mannose),

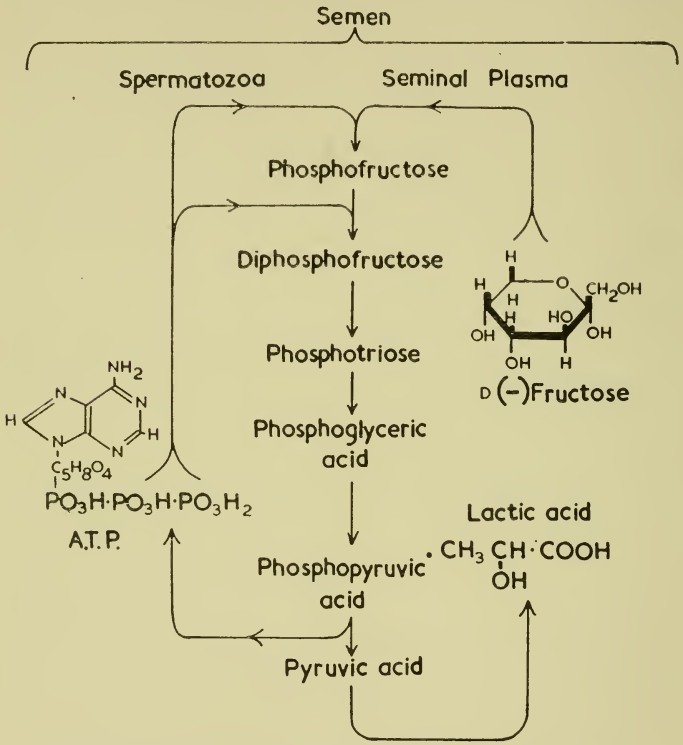


FIG. 15. *Diagrammatic representation of fructolysis in semen.*

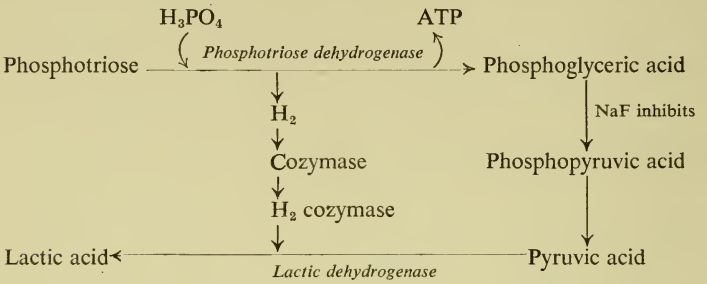
sodium fluoride, and ATP, there is on incubation a rapid disappearance of half of the readily-hydrolysable phosphorus of ATP and formation of adenosinediphosphate (ADP) and 6-phosphohexose. Of course, without added ATP and fluoride, 6-phosphohexose does not accumulate but the degradation of sugar continues uninterruptedly to its final stage, i.e. the formation of lactic acid.

If either glucose alone or fructose alone is used as substrate, the rate of lactic acid production is the same (Mann and Lutwak-Mann, 1948), but if washed spermatozoa are made to act on a 1 : 1 mixture of glucose and fructose, then the rate of fructose utilization becomes much less than 50% (Mann, 1951*b*). This 'sparing effect' of glucose on the utilization of fructose is probably due to the competitive inhibition of sperm hexokinase. Slein, Cori and Cori (1950) have shown that when brain or yeast hexokinase acts upon ATP and on an equimolar mixture of glucose and fructose, the aldosugar is phosphorylated much more rapidly than the ketosugar. Under natural conditions only fructose is present in whole semen, but not glucose, so that the possibility of the latter interfering with fructolysis does not arise. But there is preferential utilization of glucose in the accessory glands, directly responsible for the accumulation of fructose, and this may be due to a stronger affinity of hexokinase for glucose than for fructose. A competition for hexokinase, between glucose and fructose, is also consistent with the observation that in bull semen incubated with an egg-yolk-diluent, the initial rate of fructolysis is temporarily retarded (Vantienhoven, Salisbury, VanDemark and Hansen, 1952), as is also the case in semen incubated with cow follicular fluid (Lutwak-Mann, 1954); both egg-yolk and follicular fluid contain glucose.

The phosphohexose formed from fructose as a result of hexokinase activity is 6-phosphofructofuranose; in the case of glucose the product is 6-phosphoglucopyranose. The latter, however, is readily converted to 6-phosphofructose by phosphohexose isomerase, and from this stage onwards, the enzymic degradation of glucose and fructose is identical. The chain of events which in whole semen leads from fructose to lactic acid, is diagrammatically depicted in Fig. 15. In the normal course of fructolysis, 6-phosphofructose is phosphorylated by ATP in a reaction catalysed by phosphofructokinase, to yield 1 : 6-diphosphofructose and ADP; disphosphofructose is next split by zymohexase into two molecules of phosphotriose. Like the action of phosphofructokinase, that of zymohexase was demonstrated directly in spermatozoa (Mann, 1945*b*).

The subsequent steps in sperm fructolysis are analogous to the corresponding phases in muscle glycogenolysis and blood glucolysis, and involve the participation of cozymase (diphosphopyridine

nucleotide) which as Winberg (1941) showed, is a characteristic intracellular constituent of spermatozoa. The cozymase-catalysed phase of fructolysis consists of two closely interwoven oxidoreduction processes (Mann, 1945*b*; Mann and Lutwak-Mann, 1947).



The first oxidoreduction involves the oxidation of phosphotriose to phosphoglyceric acid by phosphotriose dehydrogenase, and a simultaneous reduction of cozymase to dihydrocozymase; the oxidation of phosphotriose is coupled with an esterification of inorganic phosphate and the synthesis of ATP; the oxidation product, phosphoglyceric acid, is converted by enolase to phosphopyruvic acid, the phosphate of which is transferred to ADP, thus producing pyruvic acid and ATP. The second oxidoreduction is between dihydrocozymase and pyruvic acid: dihydrocozymase is oxidized to cozymase, and pyruvic acid is reduced by lactic dehydrogenase to L(+)-lactic acid. When washed ram spermatozoa are treated with fluoride (to inhibit enolase), and incubated with added phosphotriose and pyruvate, the two oxidoreduction processes continue as usual but in addition to lactic acid there is an accumulation of phosphoglyceric acid. Iodoacetate, on the other hand, abolishes the oxidoreductions in washed sperm and thus deprives them of the ability to produce lactic acid.

The not unimpressive array of facts available from the outlined studies on spermatozoa strengthens the belief that ATP is the cardinal link between the activity of spermatozoa on the one hand, and carbohydrate metabolism on the other. In whole semen, ATP acts continually as phosphate-donor and acceptor in the course of fructolysis. The content of ATP and with it the motility of ejaculated

spermatozoa, both depend on the maintenance of the normal metabolism of fructose.

We still remain confronted with two questions to which, it is confidently hoped, further research will bring answers. One involves the as yet obscure position in the semen of animal species which lack fructose. Secondly, one wonders why nature should have chosen fructose and not glucose, as the natural substrate for sperm metabolism. At this point, conditions in another body fluid, the milk, come to mind; there, the occurrence of lactose poses a somewhat similar question. But in considering the matter, several facts must be taken into account. To begin with, if glucose and not fructose were present in semen, its concentration could hardly be expected to exceed that of blood and other body fluids. Thus, it might not be sufficient to satisfy the metabolic requirements of spermatozoa which, unlike most other animal cells, are capable of utilizing fructose anaerobically; it is worth noting that on the whole, yeasts and bacteria are also unable to consume fructose at the same rate as glucose. Presumably, this enables the spermatozoa to draw freely upon seminal fructose without, as it were, competition from other tissues. Lastly, the intimate relationship between seminal fructose and the male sex hormone must not be lost sight of; it would be rather difficult to envisage a similar dependence in the case of glucose, bearing in mind the ubiquitous occurrence and physiological function of this sugar.

CHAPTER VIII

Spermine, Choline, Ergothioneine, and certain other Bases in Semen

Spermine. Occurrence of crystalline spermine in human semen; its chemical nature and properties. Derivatives of spermine and their use in forensic medicine. Synthesis of spermine. Spermidine. Oxidation of spermine and spermidine by diamine oxidase. State of spermine in semen.

Choline. The Florence reaction in semen. Enzymic liberation of choline from precursors in semen. Phosphorylcholine and glycerylphosphorylcholine. Physiological function of free and bound choline. Choline esterase.

Ergothioneine. Isolation of ergothioneine from the boar seminal vesicle secretion. The function of seminal ergothioneine and its behaviour towards sulphydryl-binding substances. Biogenesis of ergothioneine.

Creatine and creatinine. Occurrence in mammalian semen, and in the sperm and gonads of invertebrates. Phosphocreatine and phosphoarginine.

Adrenaline and noradrenaline. Occurrence in semen and accessory organs. Enzymic oxidation. Pharmacodynamic properties.

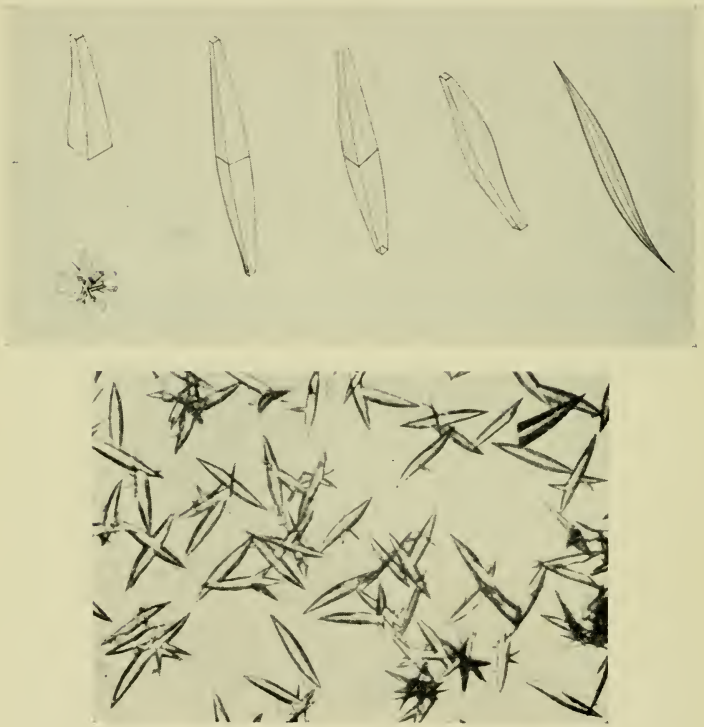
Among the chemical characteristics which distinguish semen from other tissues and body fluids is the occurrence of certain nitrogenous bases, largely betaines, which are rarely found elsewhere in the animal body. Of these, spermine is the oldest-known, and ergothioneine the most recently discovered.

SPERMINE

Occurrence of crystalline spermine; its chemical nature and properties

When human semen 'had stood a little while, some three-sided bodies were seen in it, terminating at either end in a point; some were of the length of the smallest grain of sand, and some were a little bigger, as in Fig. A. They were further as bright and clear as if they had been crystals.' Thus, in a letter of November 1677, addressed to the Royal Society, Antoni van Leeuwenhoek reported the discovery of the crystalline substance in semen which later became

dicta materia paucillum temporis ſteterat, in ea obſervabantur trilaterales figuræ ab utraque parte in aculeum deſinentes, quibuſdam longitudo minutiſsimæ arenæ, aliquæ aliquantu'um majores, ut fig. A. *Præterea, adeo nitidæ ac pellucidæ, ac ſi cryſtallinæ fuiſſent.*



SPERMINE PHOSPHATE

Crystals in human semen as seen (from top to bottom) by Leeuwenhoek (1677), Fuerbringer (1881) and Poehl (1898).

known as *spermine*. This was actually the letter in which Leeuwenhoek also communicated for the first time the discovery of living spermatozoa and their movement in fresh semen; it was published the following year in the *Philosophical Transactions* (Plate VII). During the 200 years which followed, the same crystalline substance was rediscovered by several investigators, most of whom were apparently unaware of either Leeuwenhoek's original, or of the others' later observations.

Vauquelin (1791) observed in a semen sample which he left standing for four days, the deposition of 'cristaux transparens, d'environ une ligned de long, très-minces, & qui se croisent souvent de manière à représenter les rayons d'une roue. Ces cristaux isolés nous ont offert, à l'aide d'un verre grossissant, la forme d'un solide à quatre pans, terminés par des pyramides très-allongées, à quatre faces.' After having studied the properties and behaviour of these crystals towards different solvents, Vauquelin concluded that 'la nature de ces cristaux est analogue à celle du phosphate de chaux ou la bas des os'. The belief that the sperm crystals consist of ordinary phosphate persisted throughout the best part of the next century. In the meantime, however, the same crystalline substance was found outside the semen in other tissues and body fluids, including sputum, leucaemic blood, liver, spleen and old pathological-anatomical preparations, so that towards the close of the XIXth century spermine was already known by no less than ten names of various distinguished clinicians, anatomists and physiologists, including, in chronological order, Charcot, 1853; Foerster, 1859; Harting, 1859; White, 1861 ('leucosine'); Friedrich, 1864; Huppert, 1864; Boettcher, 1865; Neumann, 1866; Eberth, 1869; Leyden, 1872; and Zenker, 1876. But in the end, the medical world at large restricted itself largely to the use of two names, 'Charcot-Leyden crystals' with reference to organs and sputum ('asthma crystals'), and 'Boettcher crystals' with reference to semen. Boettcher himself preferred to call the substance 'Spermatin', and regarded it as a protein; he published his paper 'Farblose Krystalle eines eiweissartigen Körpers aus dem menschlichen Sperma dargestellt' in 1865, without however, taking the trouble to mention the previous investigators.

The credit for having been the first to recognize spermine as the phosphate of a new organic base, is due to Schreiner (1878) who

succeeded in preparing a number of derivatives of spermine including the hydrochloride, but who unfortunately deduced from his analyses the wrong formula for the base, C_2H_5N. In consequence of this, spermine was confused with ethyleneamine, C_2H_4NH, and with piperazine. For years to follow, piperazine was offered by a large pharmaceutical firm in Berlin under the trade name of 'Spermin', and as late as 1903 the formula of piperazine appeared under the name of spermine in Thierfelder's *Hoppe-Seyler's Handbuch der chemischen Analyse*. A great advocate of the manifold curative properties of 'real' spermine, i.e. as isolated from human semen, bull testes, or other organs, was Alexander von Poehl, who believed in the 'action of spermine as a physiological tonic on auto-intoxications' (1893), and who is best known for the monograph *Die physiologisch-chemischen Grundlagen der Spermintheorie* which he published in St. Petersburg in 1898. Poehl's book contains the records of numerous cases ranging from scurvy to syphilis, treated, apparently successfully, with the 'Sperminum Poehl'. His pharmacological and clinical work aroused much controversy, was subjected to severe criticism, and was finally altogether rejected. Yet, it is not entirely improbable that there is some justification for Poehl's 'spermine theory'. Apart from its general pharmacodynamic properties similar to those of other biological polyamines (Guggenheim, 1940), spermine may well possess some other, more specific pharmacological activity. Administered parenterally, spermine is known to be toxic to mice, rats and rabbits (Rosenthal, Fisher and Stohlman, 1952). It has also been shown to possess bacteriostatic properties. The inhibition of the growth of *Staphylococcus aureus* by human seminal plasma can be attributed, according to Gurevitch and his colleagues (1951), to the high content of spermine in human semen. Another striking example of the growth-inhibiting action of spermine has been provided by Hirsch and Dubos (1952); following up an observation that the extraction of animal tissues with mixtures of water and ethanol yields material with tuberculostatic activity *in vitro*, these authors isolated from tissue extracts a crystalline antimycobacterial substance which they found to be identical with spermine phosphate.

Leaving aside Poehl's pharmacological observations, one must nevertheless appreciate his contribution to the chemistry of spermine.

Not only was he able to refute the mistaken belief in the identity of spermine and diethylenediamine but he was also the first to analyse correctly the gold salt and the chloroplatinate of spermine and to establish that 'the organic base which is at the bottom of these double salts would have the composition, $C_5H_{14}N_2$ (1898, 1906).

However, not until 1924 was conclusive chemical and crystallographic evidence brought forward to prove the identity of spermine isolated from semen with the base obtained by similar methods from various animal organs and also from yeast. Credit for this is due to Otto Rosenheim (1924). In 1924, Dudley, Mary Rosenheim and O. Rosenheim in England, and Wrede in Germany, concluded from the molecular weight estimations of benzoylspermine and *m*-nitrobenzoylspermine, respectively, that the molecular formula of spermine is $C_{10}H_{26}N_4$, and not as formerly assumed $C_5H_{14}N_2$.

Rosenheim and his colleagues obtained spermine in the free state as a crystalline, optically inactive substance which melts between 55° and 60°, and distills at about 150° *in vacuo* without decomposition. They also found that the base is stable in hot concentrated alkali and in boiling hydrochloric acid. Their relatively simple method of isolation depends on the steam-distillation of spermine from a strongly alkaline solution.

Derivatives of spermine and their use in forensic medicine

There are several well-defined compounds of spermine, a list of which is given in Table 22. Apart from the highly characteristic insoluble phosphate, spermine can be identified particularly easily as a picrate, which can be prepared from the free base, the phosphate, or directly from semen. Spermine picrate, like the phosphate, is extremely insoluble in water. Crystallographic analysis has shown that it is identical with the substance responsible for the so-called Barberio reaction, a chemical test of diagnostic value in forensic medicine. Barberio's (1905) test consists in the addition to semen, or to an aqueous extract from the seminal stain, of picric acid in concentrated aqueous or ethanolic solution; in the presence of picric acid, there follows within a few minutes, the formation of abundant yellow crystals, resembling in shape the crystals of spermine phosphate. The statement by Barberio that the reaction appears to be

specific for human semen as distinct from animal semen, has been corroborated by other Italian investigators, particularly by Baecchi (1912). In the experience of Littlejohn and Pirie (1908), Barberio's reaction is best carried out as follows; a small piece of the stained fabric is placed upon a glass slide and macerated in a drop or two

TABLE 22. *Chemical properties of spermine and its derivatives*

Compound	Formula	Properties
Free base	$C_{10}H_{26}N_4$	Needle-shaped, colourless, odourless crystals; easily soluble in water, ethanol and butanol, insoluble in ether, benzene and ligroin, m.p. 55–60°, m.w. 202
Phosphate	$C_{10}H_{26}N_4 \cdot 2H_3PO_4 \cdot 6H_2O$	Lenticular crystals from water. long needles from ethanol. Insol. in cold water, sol. 1 : 100 in boiling water, easily soluble in dilute acid or alkali. m.p. 240°, m.w. 504. Identical with 'Boettcher's crystals' and 'Charcot-Leyden crystals'
Hydrochloride	$C_{10}H_{26}N_4 \cdot 4HCl$	Short prismatic needles, extremely soluble in water, insol. in acetone, ether and chloroform. m.p. 300–310°
Picrate	$C_{10}H_{26}N_4 \cdot 4C_6H_3O_7N_3$	Yellow needles, become black at 242°, melt sharply with decomposition at 248–250°
Chloroaurate	$C_{10}H_{26}N_4 \cdot 4HCl \cdot 4AuCl_3$	Golden-yellow, lustrous leaflets. m.p. 225° (Wrede 218°)
Chloroplatinate	$C_{10}H_{26}N_4 \cdot 2H_2PtCl_6$	Orange-yellow, well-formed crystals. m.p. 242–245°
Picrolonate		Lemon-yellow crystals, m.p. 288–289°
Benzoyl derivative	$C_{10}H_{26}N_4 \cdot 4COC_6H_5$	Crystallizes from a solution in hot acetone on addition of ligroin, in woolly balls of fine needles. m.p. 155°, m.w. 618
Tetraflavianate	$C_{10}H_{26}N_4 \cdot 4C_{10}H_6O_8N_2S$	Crystallizes if flavianic acid is used in excess. On recrystallization from water the diflavianate is formed

of distilled water; to the extract thus obtained (concentrated by evaporation, if necessary), a very small drop of an aqueous saturated solution of picric acid is added, by means of a platinum loop; after a minute or two a cover slip is applied, and the preparation examined under the microscope; 'when fully developed, the crystals have the form either of obtuse or sharp-ended needles, or of rhombic prisms frequently crossed by a refrangent line at their equator. Sometimes crosses are formed, and more rarely stars.' (For further particulars concerning Barberio's test see Harrison, 1932.) Another derivative of spermine used in medico-legal laboratories is spermine flavianate; this crystalline compound forms the basis of Puranen's reaction (Puranen, 1936; Berg, 1948).

Synthesis of spermine

The final elucidation of the chemical structure of spermine was achieved in 1926. The existence of two chains *N—C—C—C—N* and of one chain *N—C—C—C—C—N* in the spermine molecule was inferred by Dudley, Rosenheim and Starling from the identification of pyrrolidine and tetramethyltrimethylene diamine as degradation products of spermine hydrochloride and decamethyl spermine, respectively. In Wrede's laboratory, the presence of two 3*C* chains and one 4*C* chain was established the same year as a result of studies on split products obtained from spermine by oxidation with molecular oxygen in presence of copper. The final proof was provided by the English investigators when they accomplished the synthesis of spermine and showed it to be, α,δ-bis [γ'-aminopropylamino]-butane:



Spermine

The oxidation of spermine gives rise to a volatile base associated with the characteristic odour of semen. The appearance of the semen-like odour during treatment of spermine chloroaurate with metallic magnesium, first described by Schreiner (1878), probably involves also an oxidation. The base is volatile in steam, and forms a crystalline hydrochloride and chloroaurate (m.p. 204–206°); it is probably identical with *N*-γ-aminopropylpyrroline.

Spermidine

Dudley, Rosenheim and Starling (1927) also succeeded in the isolation of spermidine, a base present in the mother-liquor after separation of spermine phosphate; spermidine phosphate is much more soluble than spermine phosphate and crystallizes from the 25% ethanolic mother-liquor, after the removal of spermine phosphate, when the concentration of alcohol is increased to 50%. The properties of spermidine are similar to those of spermine. It gives the same pyrrole reaction and behaves in an identical manner towards precipitating reagents including phosphotungstic acid. Spermidine is optically inactive, and yields like spermine, the semen-like odour when a solution of its chloroaurate is treated with magnesium. The structural formula of spermidine, proved by synthesis, is that of α-[γ′-aminopropylamino]-δ-aminobutane:



Spermidine

The close chemical relationship of spermine and spermidine suggests that the two bases may be related metabolically. Little, however, is as yet known about the biogenesis and metabolism of either of these two substances. So far as spermine in human semen is concerned, there can be little doubt that its high concentration which is of the order of 50–250 mg./100 ml., is due chiefly to the prostatic secretion. Fuerbringer showed in 1881 that the prostatic gland contributes by far the greatest part of seminal spermine; on addition of two drops of 1% solution of $(NH_4)_2HPO_4$ to ten drops of freshly collected prostatic fluid, Fuerbringer observed an almost instantaneous formation of Boettcher's crystals; the examination of secretions of the other accessory organs gave a negative result. The concentration of spermine in the human prostate is subject to variations but exceeds that of any other organ. This follows both from Harrison's findings (1931) as well as from the survey carried out by Hämäläinen (1947) who determined spermine as flavianate after precipitation from trichloroacetic acid extracts; the highest values obtained by the Finnish investigator in the different organs (expressed as mg. spermine phosphate per 100 g. tissue, wet weight)

were, prostate 456, pancreas 77, adrenal 58, liver 43, spleen 40, testis 29, ovary 9. It is doubtful if Fuerbringer's (1886) belief in the 'vitalizing' effect of the prostatic secretion upon spermatozoa could be applied to spermine as such; Harrison (1931, 1933) was unable to detect any activating influence of spermine phosphate on human spermatozoa. In contrast to human, bull semen contains no spermine; this is not surprising in view of the absence of a true functional prostate in the latter species.

Oxidation of spermine and spermidine by diamine oxidase

Spermine and spermidine, both undergo oxidation in the presence of diamine oxidase, an enzyme of which there is about a hundred times more in human seminal plasma than in blood serum (Zeller, 1941); this finding together with observations by earlier investigators who found that the oxygen uptake of human semen is linked with the seminal plasma rather than the spermatozoa, led Zeller and Joël (1941) to suggest that the oxygen consumption in human semen is mediated chiefly by the spermine-diamine oxidase system. This requires further experimental proof.

State of spermine in semen

An interesting but as yet unsolved problem relates to the state of spermine in freshly voided semen. Some investigators envisaged the possibility that spermine occurs already in fresh semen as a phosphate salt which being poorly soluble, separates from the semen in the characteristic shape of Boettcher's crystals. However, in freshly ejaculated human semen there is not enough inorganic phosphate to combine with all the spermine and the content of inorganic phosphate increases on standing owing to the breakdown of phosphorylcholine (see p. 170). Furthermore, it has been the experience of all those who tried to obtain crystalline spermine phosphate from semen, that a successful crystallization can best be achieved with semen which has been allowed to stand for at least a few hours after ejaculation or by following Fuerbringer's recommendation and treating it with additional phosphate. Fuerbringer's (1881) interpretation of his own findings was that the basic component of Boettcher's crystals in ejaculated semen originates in the

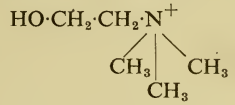
prostatic secretion, whereas the phosphoric acid is derived from some other source. Recent advances in this field favour this hypothesis and indicate that the formation of spermine phosphate takes place only after the ejaculation, as the outcome of a reaction between spermine which is contributed by the prostatic secretion, and phosphoric acid, which accumulates gradually through the action of the seminal phosphatases upon phosphorylcholine and perhaps also upon some other organic phosphorus compounds.

CHOLINE

The Florence reaction in semen

Florence, working in the laboratory of forensic medicine in Lyons, made the following observation in 1895; if material stained with human semen is extracted with water, and a drop of this extract is mixed on a microscopic slide with a strong solution of iodine in potassium iodide (2·54 g. I_2, 1·65 g. KI, 30 ml. water), the microscopic field is quickly filled with a mass of brown crystals which resemble closely Teichmann's crystals of haemin. Florence's treatise 'Du sperme et des taches de sperme en médecine légal' (1895/96) created much interest in forensic medicine and led promptly to the recognition of his test as a useful means for the identification of seminal stains. At first, a hypothetical substrate called 'virispermine' was held responsible for the formation of 'iodospermine' in the Florence reaction, but later on other substances came under investigation, including choline. All doubts concerning the nature of the Florence's reaction product were finally dispelled when Bocarius (1902) succeeded in converting 'iodospermine' preparations obtained from human and stallion semen, into a crystalline platinum compound which contained 31·62% Pt and was identical in every way with pure choline platinum chloride (31·64% Pt). Staněk's work in Prague (1905, 1906) had shown that the iodine compound formed in Florence's reaction was a water-insoluble periodide of the composition of an enneaiodide, corresponding to the formula $C_5H_{14}NOI{\cdot}I_8$. The method developed by Staněk for the quantitative determination of choline depended on the analysis of nitrogen (Kjeldahl) in the periodide precipitate; the more recent quantitative

method of Roman (1930) is based on the same principle but involves an analysis of iodine instead of nitrogen



Choline

Compared with other animal tissues and body fluids, semen ranks as one of the richest sources of choline. It owes its high choline content to the seminal plasma and not to spermatozoa as such. In rat, Fletcher, Best and Solandt (1935) found the following distribution of *total* choline (mg./100 g.): seminal fluid 514, brain 325, liver 260, pancreas 232, stomach 152, uterus 74, fat 23, blood 22. The composition is similar in other species, including man, where values exceeding 2000 mg./100 ml. semen have been observed. This may explain a statement by Marcille (1931) that a positive Florence reaction can be obtained with dried human semen even when it is diluted with 1000 parts of water. However, there is no general agreement about the sensitivity of Florence's reaction. In fact, many investigators have criticized the reaction, mainly because the same specimen of semen will occasionally give a negative reaction at first, and a positive result later. This peculiar behaviour of human semen was elucidated by Kahane and Lévy (1936, 1937) who discovered that human semen examined immediately after ejaculation contains practically no free choline, but that choline accumulates in semen gradually on standing, as illustrated by the following experiment: from 3·5 ml. semen mixed with 20 ml. water, consecutive 2 ml. samples were withdrawn and deproteinized by boiling with 9 ml. ethanol for 2 minutes; the quantitatively collected filtrates were evaporated, the residues extracted with dry ether and redissolved in water; choline was precipitated from the aqueous extracts with the Reinecke reagent and determined bromometrically. Results, which

TABLE 23. *Choline in human semen* (Kahane and Lévy, 1937)

Time after ejaculation	2 min.	10 min.	1 hr.	6 hr.	22 hr.	48 hr.	120 hr.
Choline liberated (mg. per 100 ml. semen)	70	860	1600	2120	2030	2500	530

are given in Table 23, show a sharp increase in the choline content of human semen during the first hour of incubation, and the relatively slow accumulation during the next 47 hours; the terminal decline is probably due to bacterial contamination.

Enzymic liberation of choline from precursors in semen

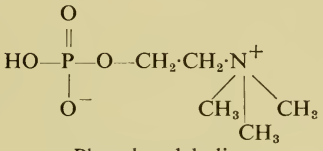
Following up their observation that choline accumulates in semen only after the ejaculation, Kahane and Lévy demonstrated the presence in fresh semen of a 'precurseur de la choline' which yields free choline as a result of hydrolysis which takes place in semen on standing. Apart from the seminal plasma itself, they found the choline precursor in various reproductive organs, including the testis of bull, boar, ram, stallion, rabbit and guinea-pig, the seminal vesicle of stallion and guinea-pig, and the epididymis of boar and ram, but not the prostate of dog, stallion or ram. However, the prostate, particularly that of dog, was found to be rich in the enzyme which splits off choline from the precursor. In a series of studies, Kahane and Lévy (1938, 1945, 1949) have shown that the precursor is a water-soluble compound ('choline hydrosoluble combinée') which behaves like glycerylphosphorylcholine, and yields on incubation with prostatic extracts a mixture of free choline and inorganic phosphate; the quantity, however, of liberated choline was found to be far in excess of the simultaneously appearing inorganic phosphate.

Phosphorylcholine and glycerylphosphorylcholine

The nature of the phosphorus compounds in semen which yield choline after ejaculation, was investigated by Lundquist (1946, 1947*a*, *b*) and by Diament, Kahane and Lévy (1952).

In human semen deproteinized freshly with trichloroacetic acid, the Danish investigator found 110 mg. acid-soluble P/100 ml., including 10 mg./100 ml. of inorganic phosphate. On neutralization with barium hydroxide and precipitation with 2 vol. of ethanol, he recovered 60–70% of the phosphorus in the filtrate and from this he obtained by precipitation with mercuric chloride a fraction containing nitrogen and phosphorus in a ratio of approximately 1 : 1. The phosphorus compound thus separated was found to be very

resistant to acid hydrolysis and no choline was set free from it after an hour's hydrolysis with N-H_2SO_4 (100°), long enough for glyceryl-phosphorylcholine, to release all its choline in a free form. On the other hand, under the influence of the prostatic secretion the compound yielded equivalent amounts of choline and inorganic phosphate. All these facts pointed to the identity of the compound with phosphorylcholine, a substance previously isolated from beef liver (Inukai and Nakahara, 1935). Lundquist sought to obtain proof by preparing the calcium salt; this he found to be identical with the calcium salt of pure phosphorylcholine, $C_5H_{13}O_4NPClCa \cdot 4H_2O$, obtained synthetically by the method of Plimmer and Burch (1937).

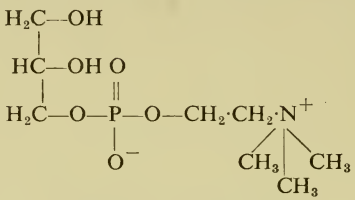


Phosphorylcholine

The distribution of phosphorylcholine in the human reproductive organs has not been investigated in detail, but Huggins and Johnson (1933) have good evidence that the bulk of the phosphorus present in the human seminal plasma is derived from the vesicular secretion. From this Lundquist infers that phosphorylcholine is formed in the seminal vesicles, and that the dephosphorylation is initiated at ejaculation, as a result of contact between the prostatic secretion which contributes the 'acid' phosphatase, and the vesicular secretion which provides the substrate; the optimum pH for the dephosphorylation of phosphorylcholine by the prostatic phosphatase measured in acetate buffer solutions is about 6·3 (Lundquist, 1947*a*, *b*). It is of some interest to recall here the claim put forward by Kutscher and Sieg (1950) that preparations of both the 'acid' and the 'alkaline' phosphatases contain pyrophosphorylcholine as a characteristic constituent. However, Roche and his colleagues (1952) were unable to detect any cophosphatase activity in pure, synthetically prepared pyrophosphorylcholine.

The possibility that compounds other than phosphorylcholine may act as precursors of free choline in semen was indicated already by the earlier observation of Kahane and Lévy that the quantity of

choline liberated after ejaculation exceeds considerably the simultaneously formed inorganic phosphate. Following up this observation, the French investigators accomplished in 1952 the isolation of a second natural precursor of choline, namely glycerylphosphorylcholine, from the seminal vesicle secretion of rats; the isolation and identification was performed as a ferric chloride compound (Diament, Kahane and Lévy, 1952, 1953). A similar result was obtained by Lundquist (1953) from his studies on the secretions of the seminal vesicles in rat and guinea-pig and the glandula vesicularis of rabbit.



Glycerylphosphorylcholine

Physiological function of free and bound choline

The occurrence of choline, phosphorylcholine and glycerylphosphorylcholine in semen and in the accessory secretions naturally raises the problem of their physiological function. One possibility which merits serious attention, is that these compounds may be bound up specifically with the metabolism of phospholipids in either the male accessory organs or in the spermatozoa.

The general importance of choline in the lipid metabolism of animals was first brought to light in 1932 when Best and his co-workers demonstrated that the appearance of the 'fatty livers' in rats fed a choline-deficient, high-fat diet, could be prevented by dietary supplements of choline. Researches which followed established two principal functions of choline, the lipotropic activity and the stimulating action on the turnover of phospholipids. In 1939, du Vigneaud and his co-workers discovered that choline is an important dietary source of methyl groups for the living animal, and this led to the recognition of choline as a participant in transmethylation processes. These three fundamental functions probably represent the clue to the understanding of the manifold symptoms associated with choline deficiency. Among the various manifestations of

choline deficiency those concerned with reproduction are particularly striking; choline is known, for example, to be essential for egg production in the chicken, as well as for normal lactation and nutrition in rats.

The role of choline in transmethylations is linked with the presence of the trimethyl quaternary nitrogen. It is worth noting, however, that while the phenomenon of transmethylation is common to a whole group of compounds bearing labile methyl groups, the lipotropic activity is restricted to choline and a few closely related derivatives. One of the lipotropically active derivatives is phosphorylcholine (Welch and Welch, 1938), and there is some evidence that the incorporation of choline into phospholipids proceeds via phosphorylcholine (Wittenberg and Kornberg, 1953).

A further possibility regarding the function of choline in semen comes to mind; choline and its derivatives belong to a group of substances endowed with well-defined pharmacological properties, and it is not improbable that the base itself or one of its compounds may exert some pharmacodynamic effects either on the spermatozoa or, perhaps, on some parts of the male or female reproductive tract. When assayed by Goldblatt (1935*b*) on the *m. rectus abdominis* of the frog, 1 ml. human seminal plasma exhibited roughly the same activity as 1μg. acetylcholine. There is, however, no chemical evidence to show that the substance in seminal plasma, responsible for this activity is in fact, acetylcholine.

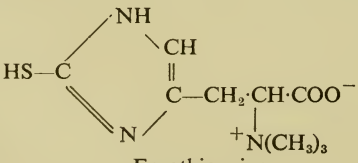
Choline esterase

It has been claimed that sperm motility is somewhat increased by acetylcholine, and depressed by eserine, but this effect has never been analysed quantitatively and requires confirmation. There is, on the other hand, sufficient evidence to show that semen contains choline esterase as a normal constituent. In human semen, the concentration of choline esterase was found to be low. Zeller and Joël (1941) using the manometric method, and employing a rather high concentration of acetylcholine as substrate, found that the quantity of acetic acid liberated by 1 ml. semen in 1 hr. is equivalent to not more than 70 μl. CO_2, as compared with 3600 μl. in blood serum and 38000 μl. in brain; moreover, the bulk of activity was derived from the seminal plasma and not from the spermatozoa. Boar

semen, on the other hand, has been found by Sekine (1951) to be highly active, the activity being more concentrated in the spermatozoa than in the seminal plasma. According to this author, boar spermatozoa, both epididymal and ejaculated, possessed choline esterase activity as high as that of brain, whereas the seminal plasma was only one-third as active as human blood serum. Boar spermatozoa, although highly active against acetylcholine, were found at the same time to be completely inactive against benzoylcholine which suggests that their choline esterase is of the 'true' or 'specific' type. Results obtained on ram semen (Legge and Mann, unpublished data) lead to a similar conclusion; ram spermatozoa exhibited a high activity at low concentrations of acetylcholine but were poorly active at high substrate concentrations, and hydrolysed efficiently acetyl-β-methylcholine but were ineffective against benzoylcholine. A study was also made at the same time of the distribution of the enzyme between the sperm-heads and -tails, using ram spermatozoa disintegrated with glass beads in the Mickle mechanical shaker (see p. 87). Choline esterase occurred mainly in the tail fraction.

ERGOTHIONEINE

Ergothioneine was first discovered by Tanret (1909) who isolated it from rye ergot. Two years later, Barger and Ewins (1911) identified the new substance as a betaine of thiolhistidine (β-2-thiolglyoxaline-4(5)-propiobetaine). The final confirmation of the structure was provided by Heath, Lawson and Rimington (1950, 1951) who succeeded in synthesizing ergothioneine from 2-thiolhistidine; the latter is an amino acid which so far has never been found in nature but was prepared synthetically by Harington and his co-workers (Ashley and Harington, 1930; Harington and Overhoff, 1933), and shown by Neuberger and Webster (1946) to be unable to replace histidine as a growth-promoting factor in animals.



Ergothioneine

Ergot from which ergothioneine has been obtained in yields varying from 65 to 260 mg./100 g., remained the only natural source of this base until Hunter and Eagles (1925, 1927) isolated from pig blood a crystalline substance, named at first 'sympectothion', which gave with phosphotungstic and arsenophosphotungstic acid reagents the same blue colour as uric acid. Quite independently, a blood constituent with similar properties, named 'thiasine', was obtained by Benedict, Newton and Behre (1926). Somewhat later, both sympectothion and thiasine were shown to be identical with ergothioneine (Newton, Benedict and Dakin, 1926; Eagles and Johnson, 1927). Blood ergothioneine, or 'thioneine' as it is sometimes called, occurs only in the erythrocytes and is not found in the plasma. In human blood there is no more than about 2 mg./100 ml., but in the pig there may be as much as 26 mg./100 ml. ergothioneine (Hunter, 1951). Of the existing methods for the determination of ergothioneine that of Hunter (1928, 1949), based on the diazo reaction, is the most sensitive, specific and accurate.

Isolation of ergothioneine from the boar seminal vesicle secretion

It has been known for quite a while that protein-free extracts from semen exhibit a marked reducing power towards iodine, silver nitrate, 2 : 6-dichlorophenol-indophenol, and potassium permanganate in the cold, and that this property is due to substances secreted in the seminal vesicle fluid. It has been mostly taken for granted however, that the reducing power of semen is due to ascorbic acid, particularly in the case of bovine and human semen (see p. 23) and no attempt was made to strengthen this assumption by a chemical identification. In 1951, Leone and Mann undertook to purify the reducing substance from the boar seminal vesicle secretion, which being available in relatively large quantities, appeared to offer a convenient source of starting material. It was noticed in the course of the purification procedure that the reducing power went parallel with three other chemical properties of the boar vesicular secretion, (i) ability to reduce phosphotungstic acid to a blue reaction product, (ii) a strongly positive diazo reaction, and (iii) the occurrence of organically-bound sulphur which, however, unlike that present in glutathione, cysteine or methionine, could be oxidized and readily split off as inorganic sulphate, by the addition of mild

oxidizing agents such as ferric chloride or bromine water. These facts suggested that the reducing substance under investigation may be the imidazole base ergothioneine. Further purification led to the isolation from 1300 ml. of boar vesicular secretion of 0·48 g. crystalline material which was finally identified by analysis of sulphur (14·0%), nitrogen (18·3%), carbon (47·1%) and hydrogen (6·6%), and by other chemical means, as pure ergothioneine, $C_9H_{15}N_3O_2S$.

With the isolation of ergothioneine from the boar vesicular secretion and boar semen, a rather unsuspected and abundant source of this sulphur-containing base in nature has been discovered. Unlike in blood, however, ergothioneine in the vesicular secretion is an extracellular constituent. Moreover, the concentration of ergothioneine in this accessory secretion is much higher than in blood. In samples from twenty boars of the Large White and Essex variety, we found from 29 to 256 mg./100 ml.; the average was 79 mg./100 ml.; in boar semen itself the concentration is about 15 to 20 mg./100 ml., but pig urine (boar and sow), and the foetal fluids contain practically no ergothioneine (Mann and Leone, 1953).

The function of seminal ergothioneine and its behaviour towards sulphydryl-binding substances

If ergothioneine possesses a specific physiological role in boar semen, this may well be linked, through its reducing sulphydryl groups, with a protective influence on spermatozoa. Boar semen, it must be remembered, differs from that of most other domestic animals by its exceptionally large volume and, at the same time, very low concentration of spermatozoa. Moreover, the period of time required for the completion of ejaculation is much longer in the boar than in other animals. Under storage conditions *in vitro*, the survival period of ejaculated boar spermatozoa compares on the whole unfavourably with that of ram and bull sperm.

The results of investigations by Brachet (1944) and MacLeod (1951) have brought into prominence the importance of reduced sulphydryl groups for sperm motility, and, as previously mentioned (p. 58), substances with sulphydryl groups in a reduced form, such as cysteine or reduced glutathione, protect spermatozoa *in vitro* from the inhibitory action of SH-binding reagents. It is probable that glutathione plays actually a role *in vivo* since it has been shown

to occur normally in spermatozoa (Infantellina, 1945; Tesoriere and Infantellina, 1946). Our researches (Mann and Leone, 1953) demonstrated that ergothioneine, which is a natural constituent of the seminal plasma, can counteract most efficiently the sperm-paralysing action of various thiol-reagents, including not only the mercaptide-forming and alkylating reagents but also substances such as *o*-iodosobenzoate which act by oxidizing compounds with SH-groups to the corresponding S-S derivatives. In fact, we were able to demonstrate the mutually antagonistic action of ergothioneine and *o*-iodosobenzoic acid in experiments with boar sperm taken

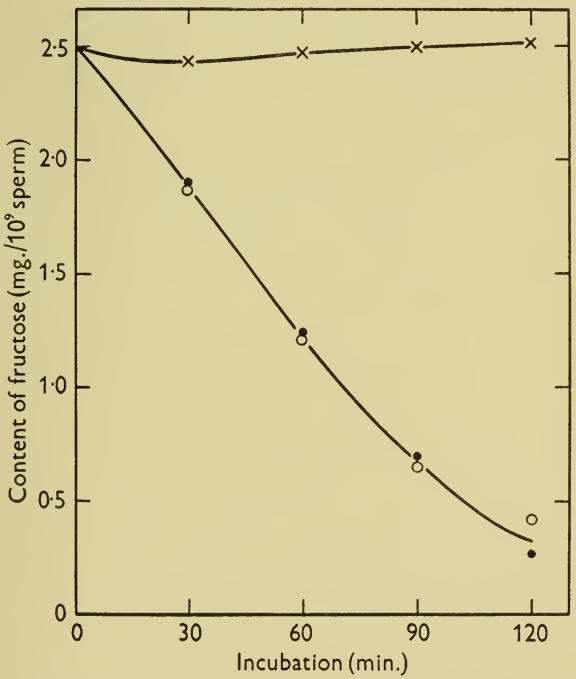


FIG. 16. *Effect of ergothioneine on boar spermatozoa;* anaerobic fructolysis at 37° in boar epididymal spermatozoa to which fructose was added, 2·5 mg. fructose/10^9 sperm; ○———○, no additions; ×———×, iodosobenzoate (10^{-3}M); ●———●, iodosobenzoate (10^{-3}M) + ergothioneine (2×10^{-3}M).

(Mann & Leone, 1953)

directly from the epididymis, in which unlike in the seminal vesicle, ergothioneine is absent. The epididymal spermatozoa were diluted with Ringer-phosphate-fructose, and the suspension divided in three equal portions; in one, serving as a control, fructolysis was measured directly, in another the reaction was allowed to proceed in the presence of 10^{-3}M-iodosobenzoate, and in the third after the addition of the same amount of inhibitor, but together with ergothioneine, the latter in a concentration of the same order of magnitude as actually found *in vivo* in the boar vesicular secretion. It can be seen from Fig. 16, that whereas the presence of iodosobenzoate alone checked the process of fructolysis, the inhibition was prevented by the simultaneous addition of ergothioneine so that in effect, the spermatozoa were able to proceed with the normal utilization of fructose.

Biogenesis of ergothioneine

The mechanism of ergothioneine formation in the boar was studied by pursuing the fate of certain orally administered compounds labelled with radioactive sulphur, ^{35}S (Heath, Rimington, Glover, Mann and Leone, 1953). It was found that inorganic sulphate or thiolhistidine failed to provide a source of sulphur for ergothioneine in the boar; in this respect, the behaviour of thiolhistidine is of particular interest, since it demonstrates again that physiologically occurring substances need not necessarily arise from compounds to which they bear a close, though purely structural, chemical resemblance. Methionine, the amino acid pivotal in biological transmethylations, was capable of supplying the sulphur for the biosynthesis of seminal ergothioneine. The spermatozoa themselves also incorporated sulphur from labelled methionine but here, the maximum radioactivity appeared several weeks later than in the seminal plasma; this time-lag is presumably due to the fact that the processes of spermatogenesis, sperm maturation, and transport through the epididymis, require substantially more time than is needed for the formation and secretion of seminal plasma in the accessory organs. By administering to a living animal a labelled compound like methionine one might be actually able to determine the time interval required for the processes of sperm formation and transport in the male reproductive organs. When synthetic

^{35}S-labelled ergothioneine was fed to the boar, some of it was excreted, unchanged, in the semen. This provides interesting evidence of the passage into semen of a substance absorbed from the alimentary tract.

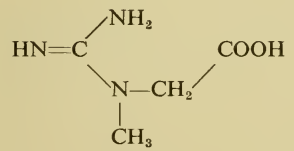
Since ordinary fodder contains no ergothioneine, there remained the possibility of its microbial formation in the digestive tract. This, however, was not borne out by an experiment in which aureomycin was fed to a boar, 1 g. daily for 24 days, but did not affect in any way the level of ergothioneine in semen.

Human semen, and that of certain other mammals so far investigated, was found to contain only a trace or no ergothioneine. In the bull, ram, and in man, the considerable reducing power of the seminal plasma towards dichlorophenol-indophenol is derived partly from ascorbic acid, but partly also from other reducing substances which await proper identification (Mann and Leone, 1953). An interesting approach in this direction was made by Larson and Salisbury (1952, 1953) who reported on the presence in bull semen of an as yet unidentified reducing substance characterized by a positive reaction with sodium nitroprusside, and of sulphite.

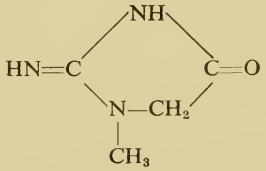
CREATINE AND CREATININE

Occurrence in mammalian semen, and in the sperm and gonads of invertebrates

One of the earliest references to the presence of creatine and creatinine in male reproductive organs is to be found in a paper by Treskin who in Hoppe-Seyler's laboratory in 1872, isolated 0·16 g. pure creatinine from two pairs of bull testes. In 1923, Steudel and Suzuki isolated large quantities of crystalline creatinine, together with another nitrogenous base, namely agmatine, from ripe, fresh testicles of herring. Ilyasov (1933), using the colorimetric method



Creatine



Creatinine

based on Jaffe's reaction, determined the creatine and creatinine content in bull and stallion semen. The mean values which he reported for creatine and creatinine were (mg./100 ml.), 3·0 and 12·1 in the bull, and 6·2 and 3·7 in the stallion, respectively. In the boar, the apparent creatinine content has been stated to be in blood plasma 2·4, in whole semen 0·3, and in the seminal vesicle secretion 5·3 mg./100 ml. (McKenzie *et al.*, 1938).

The generally held belief that in invertebrate animals arginine occurs in place of creatine is not supported by results of chemical analyses of gonads and sperm. Greenwald (1946) found in the sperm-laden nephridia of *Echiurus* 144 mg./100 g. of apparent creatine, and 189 and 270 mg./100 g. in the testes of *Arbacia* and *Strongylocentrotus*, respectively. He succeeded in preparing substantial quantities of pure creatine and creatinine, from the testes and sperm of several invertebrates, including the sea-urchin (*Strongylocentrotus*), *Urechis caupo*, *Holothuria tubulosa*, and *Cucumaria frondosa*. In the case of the gonads of two ascidia, *Microcosmus sulcatus* and *Boltenia*, which contain chromogenic material, no creatine or creatinine could be isolated but 0·19 g. of pure betaine picrate was obtained from 150 g. of mixed gonads of *Boltenia*, indicating a concentration of at least 44 mg. of betaine per 100 g. of tissue. In the testes of arthropods, molluscs, and of a nematode (*Ascaris*), the amount of chromogenic material was so low as to indicate absence of creatine.

Phosphocreatine and phosphoarginine

The possibility that spermatozoa may contain creatine in the form of phosphocreatine was envisaged by Eggleton and Eggleton (1929) who found that the testes contain, next to skeletal muscle, the second largest concentration of phosphagen. Soon after the discovery by Parnas, Ostern and Mann (1934*a*, *b*) that extracts from skeletal muscles can synthesize phosphocreatine from creatine and phosphopyruvic acid, the same enzymic reaction was investigated in bovine epididymal spermatozoa by Torres (1935) who claimed that bull spermatozoa are definitely capable of such a synthesis. Her claim, however, has been refuted by Ivanov (1937) who failed to detect any synthesis of phosphocreatine in sperm, although he experienced no difficulty in confirming our results on muscles.

More recently, Wajzer and Brochart (1947) reported on the isolation from boar sperm of a barium-precipitable fraction containing a mixture of two phosphagens, phosphocreatine and phosphoarginine.

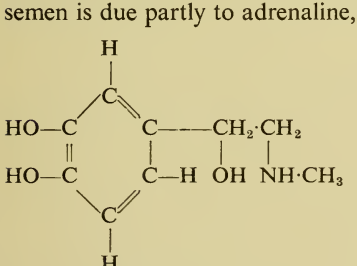
The distribution of the two phosphagens in the gonads and in sperm remains open to further investigations. An important contribution in this field was made by Greenwald (1946) who isolated phosphocreatine in the form of a calcium salt, from the testes of the carp.

ADRENALINE AND NORADRENALINE

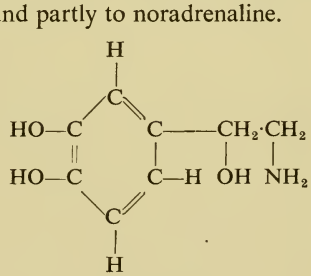
Occurrence in semen and accessory organs

Using 66% ethanol for the extraction of various tissues v. Euler found that a substance closely resembling adrenaline is present in a particularly high concentration in the prostate gland of man, dog, rabbit and guinea-pig, in the seminal vesicle of bull and ram, and in the ampulla ductus deferentis of dog, bull and ram; the amount of the active substance corresponded to 1–5 μg. of adrenaline per g. of fresh tissue.

Results obtained by Brochart (1948*a*) with the colorimetric method strengthened the view that adrenaline occurs as a normal constituent in the semen of bull (1 μg./ml.), goat (1·5–1·7 μg./ml.) and man (1·0–2·1 μg./ml.); but later, Beauvallet and Brochart (1949) came to the conclusion that in the bull at any rate, the pressor activity of semen is due partly to adrenaline, and partly to noradrenaline.



Adrenaline (epinephrine)



Noradrenaline

Enzymic oxidation

When adrenaline or noradrenaline are added to bull semen in relatively high concentrations (10–100 μg./ml.), the aerobic but

not the anaerobic, fructolysis is gradually inhibited. Brochart (1951) attributed this effect to adrenochrome and noradrenochrome which are formed aerobically through the catalytic action of the cytochrome system of bull spermatozoa upon adrenaline and noradrenaline, respectively. Adrenochrome itself, added to bull semen in amounts of 0·1–100 μg./ml., produces an instantaneous inhibition of lactic acid formation but only so long as it remains in the oxidized form; in the course of incubation with semen it becomes gradually reduced and inactive.

However, an alternative mechanism for the oxidation of seminal adrenaline may well exist since Zeller and Joël (1941) have found in extracts from the human prostate and seminal vesicle, but not in the seminal plasma, a highly active monoamine oxidase (adrenaline oxidase). The reaction catalysed by this enzyme follows the equation

$$R{\cdot}CH_2NR'+O_2+H_2O \longrightarrow R{\cdot}CHO+NHR'+H_2O_2$$

and leads to the formation of hydrogen peroxide as one of the reaction products.

Pharmacodynamic properties

An interesting synergistic relationship between adrenaline and seminal plasma was reported by Goldblatt (1935*b*) who used isolated seminal vesicles of the guinea-pig as his test object. He observed that when he added 0·5–1 ml. human seminal plasma to the medium (30 ml. oxygenated Tyrode solution) there was usually no response from the vesicles; if, however, a small amount of adrenaline was added first and a considerable interval of time allowed to elapse until the only activity of the vesicle was an occasional contraction, then the addition of the seminal plasma provoked a succession of strong contractions. Adrenaline in doses so small as to be entirely devoid of activity alone, nevertheless induced in the vesicles a condition in which the seminal plasma itself or material obtained from it by ethanolic or acetone extraction, were able to develop to the full their pharmacological activity. This behaviour of adrenaline led Goldblatt to suggest that there may be a sort of synergism between adrenaline and the seminal plasma. But it is not clear as yet, whether effects of this kind are significantly related to the function of either the male or the female reproductive system.

CHAPTER IX

Citric Acid and Inositol

Citric acid. Occurrence and distribution. Influence of male sex hormone. Citric acid in the female prostate. Metabolism and role of seminal citric acid.

Inositol. Occurrence and distribution. *meso*Inositol as a major constituent of the seminal vesicle secretion in the boar. Physiological function. Relation to other seminal constituents.

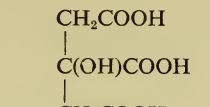
Citric acid and inositol which will be considered jointly in this chapter, are both macro-constituents of the seminal plasma. In the past these two chemical substances, much like fructose, have received attention chiefly from plant biochemists, not unnaturally, since they occur in plants in much larger quantities and more commonly than in the animal kingdom. Similarly, ergothioneine was at first associated only with the fungi, until at a much later date small amounts of it have been detected in red blood cells and more recently, it was found to be a normal constituent of boar semen.

CITRIC ACID

Occurrence and distribution

More than a century passed after Scheele's (1784) isolation of crystalline citric acid from lemon juice before this tricarboxylic acid was discovered in the animal body and identified as a major chemical constituent of milk, urine, bone and semen. The discovery in semen was made in Thunberg's laboratory at Lund, by Scherstén (1929, 1936), who noted that semen rapidly decolorizes methylene blue on addition of 'citrico-dehydrogenase', an enzyme prepared by Thunberg from cucumber seeds. This observation was strengthened by chemical identification based on isolation of crystalline citric acid and the preparation of pentabromoacetone, a derivative formed from citric acid on oxidation with permanganate and bromine, in

a reaction described in 1897 by another Swedish investigator, Stahre.



Citric acid

Scherstén enlarged his original finding by noting that citric acid in semen is derived from the male accessory organs of reproduction; in man, from the prostatic secretion, in the boar and bull, from the vesicular secretion. His findings have since been confirmed and extended by several investigators. In nine samples of human prostatic secretion Huggins and Neal (1942) recorded values ranging from 480 to 2688 mg. citric acid/100 ml., while two analyses of human seminal vesicle secretion gave 15 and 22 mg./100 ml.; in fifteen specimens of human semen, the values ranged from 140 to 637 mg./100 ml. A survey by Harvey (1951), which covered 725 specimens of human semen from 371 donors, revealed contents ranging from 0 to 2340 mg./100 ml.; the mean value of citric acid for the whole group was 479 mg./100 ml. and 12·6 mg./ejaculate. Citric acid also occurs normally in the semen of other mammalian species; a high concentration is characteristic for the bull (510–1100 mg./100 ml.), boar (130 mg./100 ml.), ram (110–260 mg./100 ml.), and rabbit (110–550 mg./100 ml.); rather lower concentrations are found in stallions (Humphrey and Mann, 1948, 1949). In some animals, e.g. the bull, ram, boar, and stallion, citric acid originates in the seminal vesicle, the same organ which also secretes fructose. In other species, however, the two substances are secreted in different parts of the male reproductive system (Table 24). In the rabbit citric acid is limited largely to the gel-portion of semen, and it is produced by the glandula vesicularis, whereas fructose, it will be remembered, is secreted also in the prostate. An even clearer separation occurs in the rat where fructose is found in the coagulating glands and in the dorso-lateral prostate, whereas citric acid is produced by the ventral prostate and the lateral lobes of the dorso-lateral prostate (Fig. 4). It is however, probable that even in species such as the bull, where citric acid and fructose are found side by

TABLE 24. *Distribution of citric acid in male reproductive organs* (Humphrey and Mann, 1949)

Species	Material	Citric acid (mg./100 g. fresh wt.)
Boar:	Secretion from Cowper's gland	0
	Prostate	38
	Epididymal semen	0
	Secretion from seminal vesicle	580
Bull:	Testis	3
	Epididymis	18
	Secretion from the seminal gland	670
	Ampullar semen	550
	Epididymal semen	0
Rabbit:	Epididymis	54
	Testis	15
	Glandula vesicularis	84
	Secretion of glandula vesicularis	834
	Prostate (I, II and III)	62
	Cowper's gland	42
	Ampulla	273
Rat:	Seminal vesicle proper	39
	Coagulating gland	0
	Ampulla	0
	Dorsolateral prostate	20
	Ventral prostate	122

side in the vesicular secretion, they may be secreted independently by different cells. A study of the bull seminal vesicle (Mann, Davies and Humphrey, 1949) has shown that the secretory epithelium is composed of three distinct types of cells, designated *A*, *B* and *C*, which appear to be concerned in the secretory processes, but react in a different manner to several histological stains. Type *B* consists of lipid-laden cells mentioned on a previous occasion (p. 133) whereas *A* and *C* cells contain no lipid, but still differ materially from each other, in so far as staining is concerned. It remains for future histochemical studies to establish their specific secretory function.

Influence of male sex hormone

There is a close relationship between the formation of citric acid in the male accessory organs and the activity of the testicular hormone (Humphrey and Mann, 1948, 1949). Following castration, citric acid gradually disappears from the accessory gland secretions but reappears on implantation or injection of testosterone. In this respect, it behaves like seminal fructose, except, however, that in some animals (e.g. rabbit) the postcastrate disappearance and the hormone-induced reappearance of citric acid in the seminal plasma is not as prompt as that of fructose. The 'citric acid test' which depends on the relationship between the formation of citric acid and androgenic activity, has been successfully used in conjunction with the 'fructose test', for the study of certain endocrinological problems, such as the time relationship between spermatogenesis and the onset of secretory function in male accessory organs (Mann, Lutwak-Mann and Price, 1948; Mann, Davies and Humphrey, 1949; Mann, 1954); formation of citric acid in subcutaneous transplants from accessory gland tissues (Lutwak-Mann, Mann and Price, 1949); and determination of androgenic activity in ovarian hormones (Price, Mann and Lutwak-Mann, 1949, 1954).

In castrated rats, a direct relationship exists between the dose of injected testosterone and the response of the seminal vesicle to produce citric acid (Mann and Parsons, 1950). This makes it possible to utilize the determination of citric acid, like that of fructose, as a sensitive and quantitative assay of androgen (Mann and Parsons, 1950). Removal of the hypophysis produces the same end-result as castration and again, the secretion of citric acid by the glandula vesicularis of a hypophysectomized rabbit can be restored, in this case, either by testosterone or by gonadotrophin (Mann and Parsons, 1950). The 'citric acid test' was also applied in studies concerned with the influence of malnutrition on the composition of semen (Lutwak-Mann and Mann, 1950*a*, *b*, 1951; Mann and Walton, 1953). The effect of malnutrition manifests itself in a progressive decline of the citric acid level in semen and accessory gland secretion and is due to a state of so-called pseudo-hypophysectomy (see p. 148).

Citric acid in the female prostate

A gland corresponding in structure to the male prostate gland develops occasionally in the female body. It has been described in women but most studies concerning the so-called female prostate have been done with rats (cf. Price, 1944; Huggins, 1945; Mann and Lutwak-Mann, 1951*b*). In the rat this organ is located in a position similar to that of the male ventral prostate which it also resembles histologically. Ordinarily the incidence of prostate gland in the female rat is very low but by inbreeding it is possible to increase it to 80% or more. With rats from such a colony, Price, Mann and Lutwak-Mann (1949) have shown that the analogy between the female prostate and the male ventral prostate extends to the chemical character of the secretion and that, like its male counterpart, the female prostate produces citric acid, but no fructose. Injections of testosterone brought about a rapid growth of the gland, and a sharp increase in the output of citric acid. In response to daily administration of 200 μg. testosterone propionate continued for three weeks, the average weight of the female prostate rose from 2 mg. to 112 mg., and the average content of citric acid in the gland from 2 μg. to 125 μg.; in male rats of comparable age the average citric acid content of the ventral prostate was 121 μg. per organ.

Metabolism and role of seminal citric acid

It is still largely a matter of conjecture how citric acid is formed in the accessory organs. The rat seminal vesicle, which is a citric acid-producing organ is at the same time remarkable for its low content of aconitase (Humphrey and Mann, 1949), and on this ground one may be inclined to assume that perhaps citric acid accumulates because its further breakdown is prevented by the absence of this enzyme. On the other hand, however, the human prostate, which is also a citric acid-producing organ, has been found to contain aconitase (Barron and Huggins, 1946*b*). A circumstance which may bear some relation to the mechanism of citric acid accumulation in the bull seminal vesicle, concerns the presence in this gland, and in its secretion, of a heat-labile factor which inhibits the enzymic breakdown of citrate by liver tissue (Humphrey and Mann, 1949). Yet another fact, mentioned briefly in conjunction

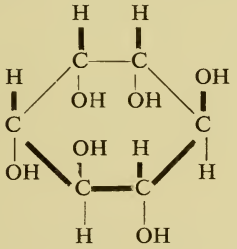
with the general chemical properties of accessory gland secretions (p. 19) concerns the high transaminase activity in the human prostate as well as in the rat ventral prostate, both of which secrete citric acid. The considerable transaminase activity together with the occurrence of free amino acids, including glutamic acid, in these two glands, point to the possibility of citric acid being formed from oxaloacetic acid which arises from glutamic acid as a result of transamination (Barron and Huggins, 1946*a*; Awapara, 1952*a*, *b*; Awapara and Seale, 1952).

As to its physiological role in semen, the available evidence does not support the view that citric acid influences markedly the aerobic or anaerobic metabolism of spermatozoa (Humphrey and Mann, 1949); thus the beneficial effect of citrate on sperm motility observed by Lardy and Phillips (1945) may be due to a cause other than direct utilization by sperm. It is conceivable that citric acid is connected with the coagulation and liquefaction of semen and with the calcium-binding capacity of seminal plasma. In this connection one may recall the finding of Huggins and Neal (1942) that citrate in human semen causes prolonged coagulation of mixtures of blood and seminal plasma, and that this delay in clotting can be effectively counteracted by calcium ions. The function of citric acid as a binding substance for calcium has been envisaged both by Scherstén (1936) and Huggins (1945) and it is certainly significant that milk and bones, both rich in citrate, also have a high calcium content. Perhaps in the absence of citric acid in the prostatic secretion, there would be an even higher incidence of calculi and stones. The possibility of a link with the hyaluronidase activity cannot be excluded, as indicated by Baumberger and Fried (1948) who found that citrate exerts a protective action against so-called antinvasin *in vitro*. Lundquist (1947*b*), however, believes that citrate may act as an activator of the prostatic 'acid' phosphatase. Lastly, let it be remembered that citric acid, in combination with potassium and sodium ions, may play a part in maintaining the osmotic equilibrium in semen. Our own studies on the boar vesicular secretion (Mann, 1954) point in this direction.

INOSITOL

Occurrence and distribution

Inositol was first discovered in 1850 by Scherer, at Würzburg, who isolated it from the mother-liquor remaining after the separation of creatine from beef meat, as a crystalline, colourless and distinctly sweet-tasting substance. He named it 'inosit' to underline its origin from muscle, and showed that its composition and properties, except for lack of reducing capacity are similar to those of a hexose. In 1887 Maquenne proved that this non-reducing and optically-inactive compound is not a sugar in the strict sense but a hexitol derived from *cyclo*hexane. In the years which followed, Bouveault (1894) and others brought forward evidence for the existence of several cyclitols derived from *cyclo*hexane; since then it became customary to define the compound from muscle as *meso*inositol, in distinction to the other isomers. The configuration of *meso*inositol was finally established in 1942, by Dangschat in Fischer's laboratory, and by Theodore Posternak, in Switzerland.



*meso*Inositol

Apart from muscle, inositol has also been isolated from urine (Cloetta, 1856), and from green beans (Vohl, 1856). Later, several plants were found to yield on extraction such very large amounts of this cyclitol, that like citric acid, *meso*inositol came to be regarded generally as a typical plant constituent, a view strengthened by the discovery of phytic acid (inositol hexaphosphate) in grain, and lipositol (a monophosphoinositol-containing phosphatide) in soya-bean. The interesting history of these and later developments in the biochemistry of inositol will be found in the monograph by

Fleury and Balatre (1947) and in the reports by Courtois, Fleury, Posternak, and Schopfer, forming part of a (1951) symposium.

Inositol as a major constituent of the seminal vesicle secretion in the boar

In 1951, in the course of investigations on ergothioneine, the author noticed that a large ethanol-precipitable fraction could be separated from the boar vesicular secretion, containing no ergothioneine, citric acid, or fructose, and almost free from sulphur, nitrogen, or phosphorus. On further purification, from 1 litre of the vesicular secretion, 18 g. of crystalline material was obtained which had a pronounced sweet taste, but was non-reducing and optically inactive. The substance had a m.p. of 225° which is that of pure *meso*inositol, and contained 40·28% carbon and 6·79% hydrogen, as against 40·11% carbon and 6·66% hydrogen, theoretically expected from inositol. The Scherer-Salkowski reaction performed with 0·1 mg. was strongly positive, and on oxidation with periodic acid the substance isolated from the seminal vesicle secretion showed a titration curve identical with that of pure *meso*inositol.

This and subsequent experiments (Mann, 1951*c*, 1954) showed that the boar vesicular secretion is the richest source of free inositol in nature, and that between 40 and 70% of the dialysable contents of this biological fluid is made up of inositol. Table 25 shows the results of chemical analyses of the vesicular secretion from five boars, carried out in each instance on fluids collected separately from the left and right gland. As can be seen, the inositol content of these fluids was 2·08 to 2·64%, the variations being much smaller than they would be in the case of fructose or citric acid. It is also of some interest to note that the left and the right seminal vesicle produced secretory fluids which were alike in quantitative composition. The average values (mg./100 ml.) based on the analyses of five pairs of secretions were: fructose 65, citric acid 381, ergothioneine 91, inositol 2414.

Inositol is restricted in its distribution to the seminal vesicle, and is not present, at any rate in appreciable quantities, in the secretions from the boar epididymis or Cowper's gland. Furthermore, it appears to be rather specific for the boar, so far as can be judged from preliminary experiments carried out with the semen of other

TABLE 25. *Individual variations in the composition of boar vesicular secretion* (Mann, 1954)

(Analyses carried out separately on the fluids collected from the left (L) and right (R) seminal vesicle of five boars, nos. I–V.)

		I	II	III	V	V
Total volume of secretion from both vesicles (ml.)		30	52	170	360	550
Total weight of the empty vesicles (g.)		32	43	160	330	370
Concentration in the vesicular secretion (mg./100 ml.)						
Fructose	(L)	17	20	56	112	118
	(R)	13	26	54	104	130
Citric acid	(L)	259	137	305	241	961
	(R)	241	141	285	235	1001
Ergothioneine	(L)	82	95	87	106	90
	(R)	82	94	81	106	88
Acid-soluble phosphorus	(L)	25	36	24	26	36
	(R)	27	38	26	24	38
Inositol	(L)	2360	2570	2640	2355	2190
	(R)	2355	2610	2640	2345	2080

species. Bull semen is poor in inositol, and human semen, according to Nixon (1952), contains usually less than 0·1%.

Physiological function

In the past, the physiological function of inositol has been associated mainly with nutrition, particularly since Woolley's (1944) important observations on the curative effect of inositol in a dietary dystrophy in mice, coupled with retarded growth and alopecia. Inositol has also been known to remedy a certain type of fatty liver in rats (Gavin and McHenry, 1941; MacFarland and McHenry, 1948), and its role as a lipotropic factor has been stressed repeatedly. An observation, however, which merits particular attention in view of its bearing on animal reproduction, concerns a peculiar disturbance in hamsters: if hamsters are raised on an inositol-deficient diet, they tend to produce dead litters or die in parturition (Hamilton and Hogan, 1944). Yet another aspect of the

function of inositol became apparent when Chargaff and his co-workers (1948) reported that in tissue cultures inositol protects dividing fibroblasts from the toxic effect of colchicine and other mitotic poisons.

It remains for future studies to determine more fully the function of inositol in boar semen. Judging from the author's own experiments there is little evidence that inositol is metabolized directly by spermatozoa. One is inclined to assume for inositol a role in the maintenance of the osmotic equilibrium in boar seminal plasma, seeing that the seminal vesicle secretion, unlike other body fluids of the pig, is almost completely devoid of sodium chloride (Mann, 1953, 1954).

Relation to other seminal constituents

The mechanism by which inositol is formed in mammals is obscure but two tentative hypotheses have been put forward in the past. One involves its formation from a derivative of phosphoinositol such as for instance, lipositol. Fischer (1945) however, believes that inositol acts in the animal body as a sort of chemical intermediary between the sugars and certain aromatic substances, or alternatively, as a reserve carbohydrate for hexoses. The close structural similarity of inositol to glucose as well as to fructose, is certainly a point which must be considered in future investigations on the origin of seminal inositol.

CONCLUDING REMARKS

A STRIKING feature of semen which did not escape Leeuwenhoek, and which has been abundantly and repeatedly confirmed since, is the extraordinary diversity of shape and structure, encountered among the spermatozoa of different species. It even led Wagner and Leuckart (1852) to state 'that one may often safely venture to infer from the specific shape of these elements the systematic position and the name of the animals investigated'. Similarly, anatomists and physiologists alike, have long accepted as natural the existence of remarkable species variations in the form and size of the male accessory glands, the organs responsible for the elaboration of that apparently indispensable adjunct of spermatozoa, the seminal plasma. It behoves us, I feel, to adopt a similarly enlightened attitude of mind towards the chemistry of semen. Is it not rather unreasonable to expect that chemical findings made with the semen of one species must needs extend to that of others? The fact that a given substance is found in substantial amounts in the semen of one species, but is missing in others, by no means detracts from its physiological value: on the contrary, it is highly probable that such species-restricted occurrence is intimately linked with some other biological characteristics, peculiar to certain, but not necessarily all, animal species.

A critical approach, free from bias, is also called for in the comparative evaluation of the morphological and chemical findings in semen. To expect, as has been done, the existence of a strict correlation between say, the fructose level in seminal plasma and sperm density in semen, is no more justifiable than to look for a relationship between, for instance, the glucose level in blood plasma and the number of red cells in blood. Similarly, although the secretion of fructose in the accessory organs depends closely upon the activity of the male sex hormone, it would be mistaken to attribute the level of fructose in semen to the influence of this hormone alone, because in reality it is conditioned by a multitude of other factors, including the general nutritional state of the body, size and storage capacity

of the accessory glands, frequency of ejaculation, volume of semen, ratio between sperm and seminal plasma, and last but not least, the blood glucose level. Above all, it is essential to bear in mind that profound changes in the composition of semen, elicited in response to drastic experimental procedures like castration or hypophysectomy, are unlikely to be equalled in extent by those encountered in hormonally deficient humans or in large domestic animals.

Having thus come to the end of my discourse, I would like to leave the last word with Leeuwenhoek; when reporting in 1677 to the Rt. Hon. the Viscount Brouncker, President of the Royal Society, upon the progress of his researches on semen, he felt it incumbent upon him to add: 'If your Lordship should consider that these observations may disgust or scandalize the learned, I earnestly beg your Lordship to regard them as private and publish or destroy them, as your Lordship thinks fit.'

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